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REVIEW

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Insights into next-generation immunotherapy designs and tools: molecular mechanisms and therapeutic prospects

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Abstract

Immunotherapy has revolutionized the oncology treatment paradigm, and CAR-T cell therapy in particular represents a significant milestone in treating hematological malignancies. Nevertheless, tumor resistance due to target heterogeneity or mutation remains a Gordian knot for immunotherapy. This review elucidates molecular mechanisms and therapeutic potential of next-generation immunotherapeutic tools spanning genetically engineered immune cells, multi-specific antibodies, and cell engagers, emphasizing multi-targeting strategies to enhance personalized immunotherapy efficacy. Development of logic gate modulation-based circuits, adapter-mediated CARs, multi-specific antibodies, and cell engagers could minimize adverse effects while recognizing tumor signals. Ultimately, we highlight gene delivery, gene editing, and other technologies facilitating tailored immunotherapy, and discuss the promising prospects of artificial intelligence in gene-edited immune cells.

Keywords Chimeric antigen receptor, Multi-targets, Logic gate, Adapter CAR, Gene delivery, Gene editing

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Introduction

Immunotherapy has inaugurated a novel era in the fight against malignancies. Among the forefront of these advancements, gene-edited immune cell therapies, particularly Chimeric Antigen Receptor (CAR)-T cell therapy, have exhibited remarkable efficacy in the battle against hematological malignancies. CAR-T cell therapy enables the specific targeting of genetically modified T cells into the tumor cells, circumventing the reliance on MHC molecules. The procedure involves the redirection editing of T cells in vitro, which are then reinjected into the patient (Fig. 1). Although most patients exhibit a favorable response and overall remission rate to CAR-T cell therapy, tumor recurrence remains a great challenge [1]. The essential contributor to treatment failure is the loss of CD19 or BCMA [2, 3] and the emergence of antigen-negative clones [4]. Furthermore, the controllability of CAR-T cell-related encephalopathy syndrome (CRES) and cytokine release syndrome (CRS) represents major obstacles to scaling up the clinical application [5, 6]. Although CAR-T cell therapy has been investigated for the potential in treating solid tumors and autoimmune diseases [7, 8], expanding CAR-T cell therapies remains challenging due to the safety, efficacy, and effectiveness concerns. The inherent limitations of autologous cell therapies, including protracted manufacturing timelines [9], prohibitive costs [10], and donor dependency [11, 12], have catalyzed a paradigm shift toward developing universal allogeneic CAR-immune cell platforms. As an alternative therapeutic strategy, allogeneic CAR-immune cell-based therapy demonstrates superior cost-effectiveness and significantly reduces patient waiting periods [13] (Fig. 1). Moreover, this approach circumvents the limitations associated with patient donor cell quality and insufficient quantity through the utilization of cells derived from healthy donor sources, including peripheral blood mononuclear cells (PBMC) [14], hematopoietic stem/progenitor cells (HSPC) [15], and induced pluripotent stem cells (iPSC) [15, 16].

Beyond CAR-T cells, next-generation immunotherapy tools encompass a diverse array of gene-edited immune cells, including CAR-natural killer (CAR-NK) cells, CAR-Macrophage (CAR-M) cells and CAR-unconventional T cells. These alternative approaches possess unique advantages in augmenting the anti-cancer activity and minimizing off-target effects, offering novel avenues for deciphering the complex code of clinical translation. Moreover, emerging cutting-edge technologies could construct more sophisticated cellular programming systems to realize the precise regulation of the multi-antigen targeting profiles, thereby yielding novel optimization strategies for the next-generation gene-edited immune cells [17]. To illustrate, the engineered CARs precisely modulate the targeting and activity of gene-edited

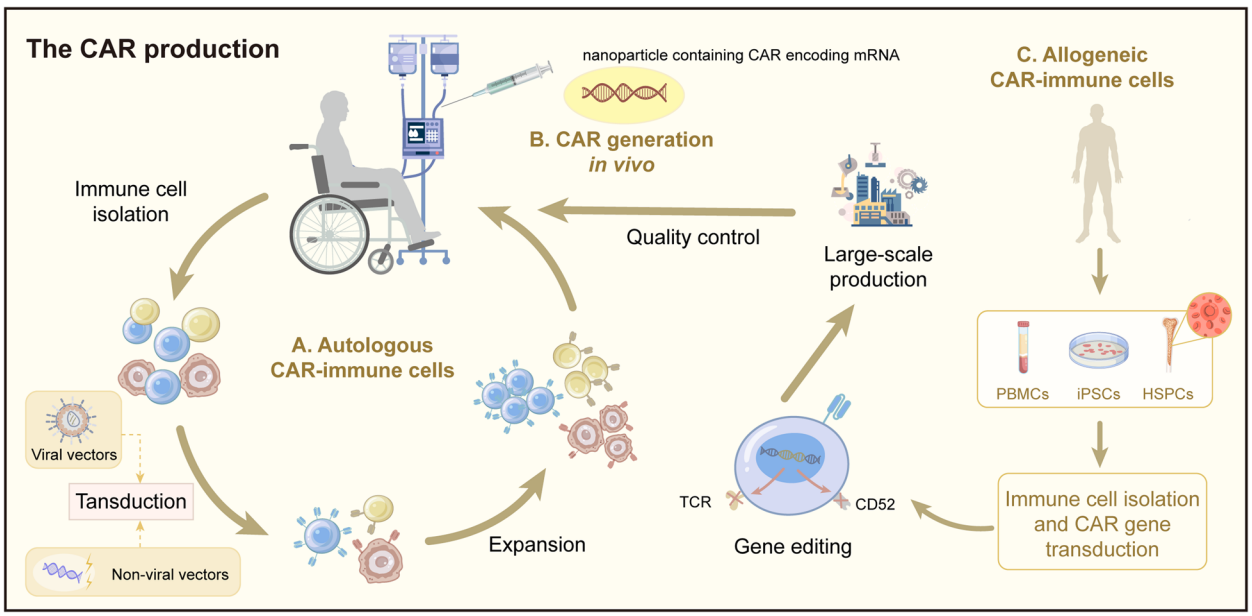


Fig. 1 CAR production. **A**. Autologous CAR-immune cells. The process includes isolating immune cell populations from the patient, followed by transduction of these cells with viral or non-viral vectors to express the CAR. After ex vivo expansion, the engineered immune cells are infused back into the patient. **B**. CAR generation in vivo. Injecting nanoparticles containing mRNA that encodes the CAR could achieve CAR expression in vivo. **C**. Allogeneic CAR-immune cells. Immune cells are isolated from healthy donors, undergo genetic editing to reduce immunogenicity and minimize immune rejection, and are then produced at a large scale with rigorous quality control before being applied in clinical settings

immune cells. Meanwhile, adapter molecules could augment their adaptability and facilitate high-specificity engagement with a diverse array of cell types [18]. We summarize the timeline of important events in multi-targeting strategies in Fig. 2.

Leveraging single-target strategies to enhance immunotherapy has proven insufficient in confronting immunological challenges, indicating that next-generation immunotherapy might necessitate multi-targeting approaches. To propel the evolution of immunotherapy, this review evaluates the merits and drawbacks of various gene-edited immune cell therapies and discusses the design and function of multi-specific antibodies and cell engagers, focusing on strategies aimed at achieving multi-targeting profiles. The review will also explore how gene delivery/editing technologies and artificial intelligence (AI) can aid the development of personalized immunotherapy.

Gene-edited immune cells

CAR-T cell

CAR-T cells, autologous/allogeneic T cells genetically transduced to express antigen-specific CAR proteins by retrovirus/lentivirus/non-viral vectors, could specifically target tumor antigens and exert potent anti-tumor effects [17, 19]. The main components of the CAR construct, including the extracellular target-binding domain (single-chain fragment variable, scFv), a hinge domain, a transmembrane domain, and an intracellular signal domain, usually determine the function of the CAR-T cell (Fig. 3A). Affinity tuning of the binder domain in CAR construct design is crucial, as it promotes optimal CAR performance in low-level targeted antigen-expressing tumors, whereas overly low or high affinities may increase off-target cytotoxicity or the risk of tumor escape [20]. The single-chain nanobody has the potential to be widely used in CAR production due to its small size, high specificity, and selective stability [21]. In contrast to classical CAR structural domains, the nanobodies exhibit reduced immunogenicity and contribute to overcoming the immunogenicity problem of linker [22]. The scFv structural domains within the nanobody-based nanosome structure maintain their affinity without exhibiting a loss, whereas the potential for cross-pairing between the VH and VL domains of two different scFv molecules in the classical CAR structure leads to decreased affinity [23]. The hinge and transmembrane domains could respectively be involved in binding target epitopes and maintaining CAR stability [24, 25]. The intracellular signal domain comprises the costimulatory domain (cluster of differentiation (CD) 28, inducible costimulator (ICOS), 4-1BB (CD137), OX40 (CD134), and CD27) [26] and activation domain (CD3ζ or Fc receptor FcεRIγ) [27], is another key factor in CAR engineering. Containing three immunoreceptor tyrosinebased activation motifs (ITAMs), the activation domain is involved in the transduction of CAR-T cell activation. In the second and third-generation CAR [28, 29], the costimulatory domain and additional domains are generally introduced to enhance T-cell activation [30]. The engineered CAR-T cells could recognize the tumor antigen directly and specifically through scFv, thereby secreting granzymes, perforin, and inflammatory cytokines to exert oncolytic impact.

The fourth/fifth generation CAR-T cells have been developed to reinforce CAR-T cell potency.

(See figure on next page.)

Fig. 2 Timeline of key events. [1] Eshhar, Z., Waks, T., Gross, G. & Schindler, D. G. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors. Proc Natl Acad Sci U S A 90, 720–724 (1993). [2] NCT00924326 [3] Hegde, M. et al. Combinational targeting offsets antigen escape and enhances effector functions of adoptively transferred T cells in glioblastoma. Mol Ther 21, 2087–2101 (2013). [4] Fedorov, V. D., Themeli, M. & Sadelain, M. PD-1- and CTLA-4-based inhibitory chimeric antigen receptors (iCARs) divert off-target immunotherapy responses. Sci Transl Med 5, 215ra172 (2013). [5] https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-blinatumomab-consolidation-cd19-positive-philadelphia-chromosome-negative-b-cell [6] Hegde, M. et al. Tandem CAR T cells targeting HER2 and IL13Ralpha2 mitigate tumor antigen escape. [7] Ruella, M. et al. Dual CD19 and CD123 targeting prevents antigen-loss relapses after CD19-directed immunotherapies. J Clin Invest. 126, 3814–3826 (2016). [8] Roybal, K. T. et al. Engineering T Cells with Customized Therapeutic Response Programs Using Synthetic Notch Receptors. Cell 167, 419–432 e416 (2016). [9] Bielamowicz, K. et al. Trivalent CAR T cells overcome interpatient antigenic variability in glioblastoma. [10] Sukumaran, S. et al. Enhancing the Potency and Specificity of Engineered T Cells for Cancer Treatment. Cancer Discov 8, 972–987 (2018). [11] Pan, J. et al. Sequential CD19-22 CAR T therapy induces sustained remission in children with r/r B-ALL. Blood 135, 387–391 (2020). [12] Liu, E. et al. Use of CAR-Transduced Natural Killer Cells in CD19-Positive Lymphoid Tumors. N Engl J Med 382, 545–553 (2020). [13] Au, K. M., Park, S. I. & Wang, A. Z. Trispecific natural killer cell nanoengagers for targeted chemoimmunotherapy. Sci Adv 6, eaba8564 (2020). [14] NCT04660929 [15] NCT04606433 [16] A first-in-human study of CD123 NK cell engager SAR443579 in relapsed or refractory acute myeloid leukemia, B-cell acute lymphoblastic leukemia, or high-risk myelodysplasia.—ASCO [17] Wang, X. et al. Allogeneic CD19-targeted CAR-T therapy in patients with severe myositis and systemic sclerosis. Cell 187, 4890–4904 e4899 (2024). [18] Wang, X. et al. Allogeneic CD19-targeted CAR-T therapy in patients with severe myositis and systemic sclerosis. Cell 187, 4890–4904 e9 (2024). [19] Zhou, J.-e. et al. Lipid nanoparticles produce chimeric antigen receptor macrophages (CAR-M) in situ for the treatment of solid tumors. Nano Today 61, 102,610 (2025)

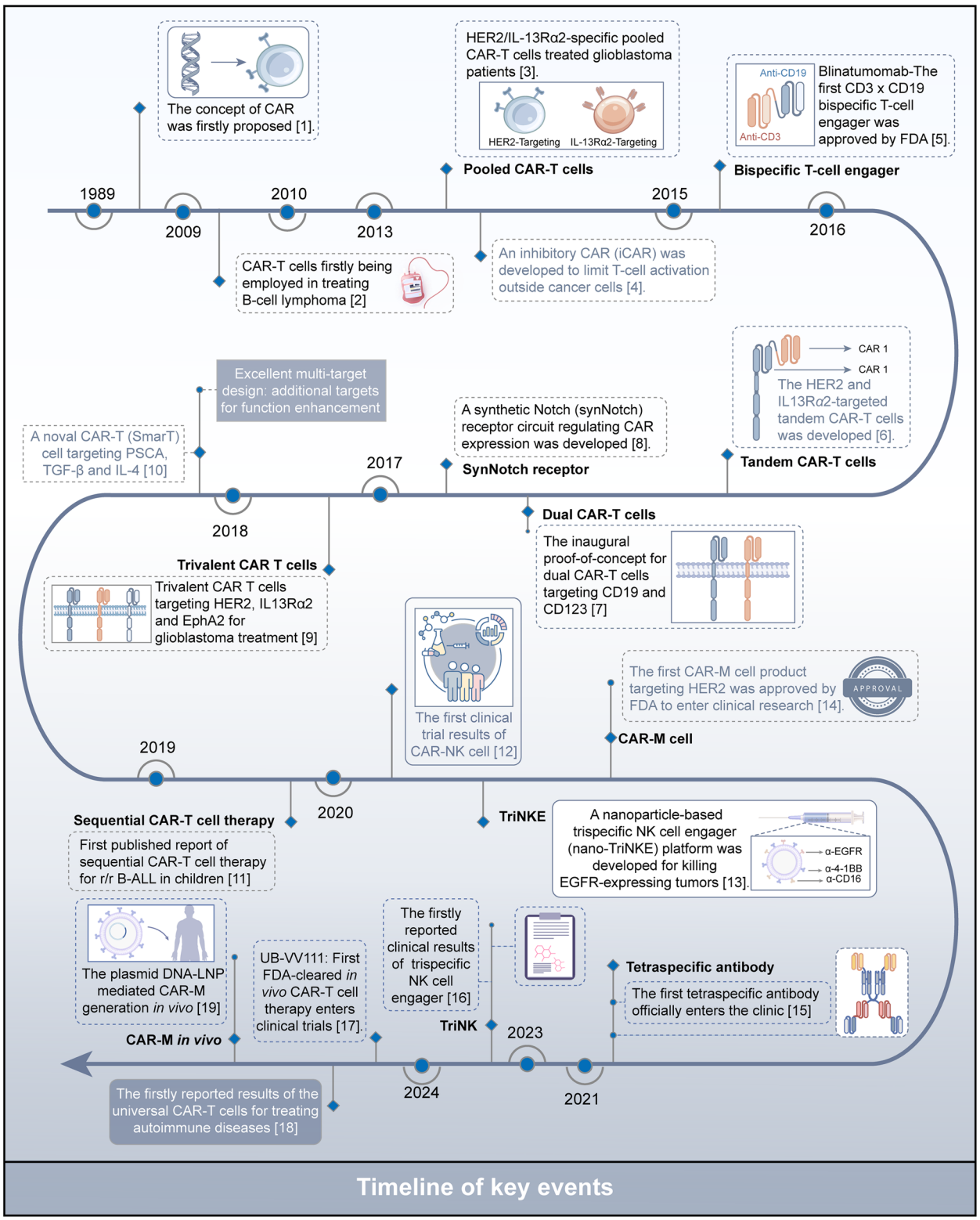


Fig. 2 (See legend on previous page.)

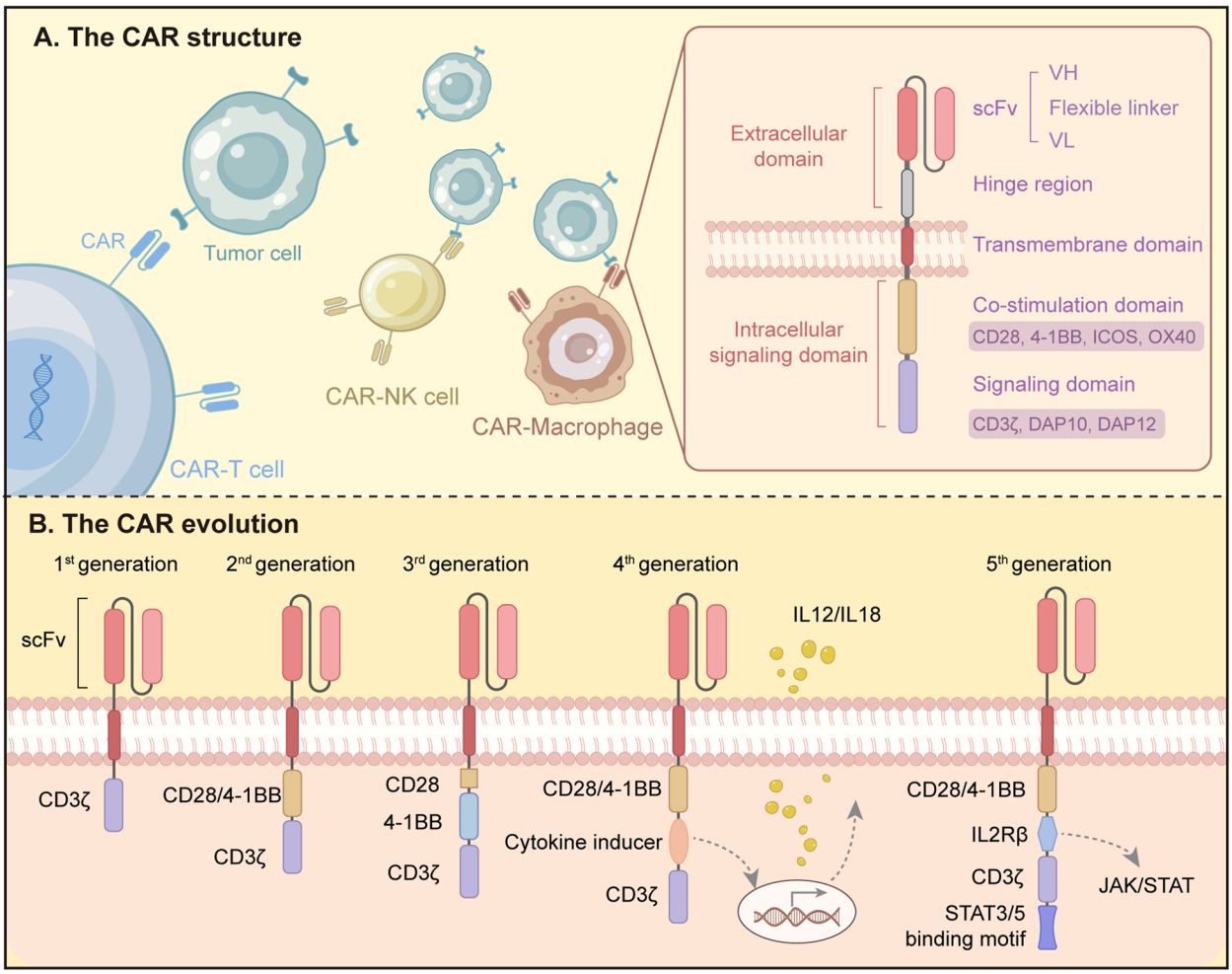


Fig. 3 Overview of CAR. **A**. Structure of CAR The structure of a CAR includes the extracellular domain, transmembrane domain, and intracellular domain. The extracellular domain comprises the single-chain variable fragment (scFv), which is composed of VH, VL, and a flexible linker, along with the hinge region. The intracellular domain includes co-stimulatory domains and signaling domains. **B**. The CAR evolution The evolution of CAR structures primarily involves modifications to the intracellular domain. First-generation CARs contain a simple CD3ε domain. The second-generation and third-generation CARs introduce one and two co-stimulatory domains, respectively. Fourth-generation CARs incorporate additional functional domains, such as those promoting cytokine release. The fifth-generation CARs integrate JAK/STAT signaling to enhance immune cell persistence and sensitivity to immunosuppressive microenvironments

Fourth-generation CAR-T cells, referred to as T-cells redirected for universal cytokine-mediated killing (TRUCKs), have been further engineered to secrete specific cytokines such as IL-12, IL-15, IL-18, CCL19, and IL-7 [31] (Fig. 3B), thereby recruiting and activating other innate immune cells to enhance anti-tumor effects. Incorporating additional co-stimulatory molecules and signaling cascade pathways, such as the JAK/STAT pathway, fifth-generation CAR-T cells are engineered to effectively combat the tumor microenvironment (TME), with enhanced persistence and cytotoxic efficiency [32] (Fig. 3B). Meanwhile, another defined fifth-generation CAR-T cells, "off-the-shelf" products designed with allogeneic CAR-T cells, show potent anti-tumor activity, markedly reducing the temporal and economic burdens associated with CAR-T cell production and expediting the commercialization pathway [33].

CAR-NK cell

NK cells, as innate lymphocyte subsets, have been an attractive strategy for immune cell therapy. Multiple

mechanisms could trigger the activation of NK cell-mediated tumor eradication, such as recognizing tumors with downregulated expression of MHC class I molecule [34, 35], binding to overexpressed NK cell activating ligands on tumor cell surface [34, 36], and antibody-dependent cell cytotoxicity (ADCC) [37]. Generally, immunoglobulin-like receptors on NK cells, killer-cell immunoglobulin-like receptors (KIR), could suppress NK cell function by binding to MHC molecules on normal cells [38]. Some tumors downregulate MHC expression to evade T cell-mediated cytolysis, permitting NK cells to exert unimpeded anti-tumor activity in the absence of inhibitory signals from "self" MHC molecules [34]. It is recognized that the "missing-self" condition constitutes the distinctive oncolytic potential of NK cells [39]. Based on the "inducing self" hypothesis, tumor cells upregulate stimulatory ligands associated with NK cell activation, which provides sufficient activating signals for NK cells to override inhibitory signals by binding to multiple activating receptors—natural killer group 2, member C/natural killer group 2, member D (NKG2C/NKG2D) [40]. Additionally, NK cells express CD16 that binds to the constant region (Fc) of the antibody to exert killing effects via ADCC [41].

CAR-NK cells could be redirected to specific antigens and induce enhanced tumor-antagonizing immunity [42]. CAR activation and endogenous receptor-mediated NK cell activation kill target cells via diverse mechanisms, such as releasing perforin and granzyme for cytolysis [43], upregulating death ligands—FAS cell surface death receptor (FAS ligand) and TNF-related apoptosis-inducing ligand (TRAIL) [36], and secreting interferon-gamma (IFN-γ) for activating other immune cells [44]. Most current CAR constructs, including CD3ζ and co-stimulatory domain for T cells, employed in CAR-NK cells are identical to those used in CAR-T cells. Several studies showed that cooperative activation may occur through the interaction of CAR with activated receptors on NK cells [45, 46]. Given the unique signaling domains intrinsic to NK cells [47], including DNAX-activation protein 12 (DAP12), DAP10, and CD244, designing optimal and specific CAR constructs for NK cells may optimize their therapeutic potential and potentiate their intrinsic cytotoxic capabilities [48, 49]. NK cell-tailored CARs have shown higher cytotoxicity against tumors and improved proliferation [50–52]. Li et al. engineered NK cells derived from iPSC to express the CAR construct, comprising the transmembrane domain of NKG2D, the 2B4 co-stimulatory domain, and the CD3ζ signaling domain [53], which endowed NK cells with potent anti-tumor activity and prolonged survival time in vivo. Notably, the replacement of scFv with NK cell activating receptors or nanobodies exhibited effective tumor-killing ability [54].

CAR Macrophage Cell

Solid tumors often suppress immune cell function and promote cell exhaustion by the inhibitory TME. In contrast to the poor infiltration of CAR-T/NK cells into tumors, macrophages represent the predominant immune cell population in the TME and possess distinctive properties of tumor infiltration [55]. Macrophages could remodel the immunosuppressive TME. For instance, macrophages could degrade fibrotic matrix in tumors by secreting matrix metalloproteinases (MMP), thereby creating a microenvironment conducive to immune cell infiltration [56, 57]. CARs for macrophages share the same overall structure as those constructed for CAR-T/NK cells, but CAR-M cells harness more appropriate intracellular signal transduction domains for their activation, such as CD147, FcRγ, multiple epidermal growth factor-like domains protein 10, adhesion G protein-coupled receptor Bai1, and myeloid-epithelial-reproductive tyrosine kinase. Introducing CARs with these specific intracellular structures could redirect macrophages, whilst enhancing phagocytosis function, facilitating infiltration ability, and counteracting the immunosuppressive effects of the TME [58–61].

Given the remarkable plasticity of tumor-associated macrophage (TAM), CAR-M cell therapy significantly enhances the viability and resilience of immune cells within the TME, potentiating anti-neoplastic effects and mitigating immunosuppressive challenges [62, 63]. Klichinsky et al. reported that introducing CARs into macrophages via chimeric adenoviral vectors enabled CAR-M cells to maintain the M1 phenotype [61]. The CAR-M cells could also repolarise M2 macrophages to M1 macrophages in the TME by secreting cytokines and chemokines [61]. Moreover, CAR-M cells possess diverse killing capacities, such as antigen-specific phagocytosis and antigen-independent phagocytosis [64, 65]. Further research may concentrate not only on the particular phagocytosis of CAR-M cells but also on utilizing the antigen-presenting and immunomodulatory functions to enhance tumor immunotherapy.

The comparison of CAR-T cells, CAR-NK cells, and CAR-M cells is summarized in Table 1.

CAR-Unconventional T cells

Conventional T cells used to introduce CAR constructs are αβ T cells, whereas non-conventional T cells are a special class of T lymphocyte subsets including natural killer T (NKT) cells, mucosa-associated invariant T (MAIT) cells and γδ T cells [66]. Unconventional T cells diverge from the MHC molecule-mediated antigen recognition paradigm and exhibit unique antigen recognition and immune functions, providing a promising platform for CAR-immunotherapy.

Table 1 Comparison between different CAR-immune cells

	CAR-T Cell	CAR-NK Cell	CAR-M
Mechanism of action	Cytotoxicity induced by CAR-mediated T-cell activation	Activation of NK cells induced by recognizing the "missing-self" condition; Antibody-dependent cell cytotoxicity; Releasing perforin and granzyme for cytolysis; Upregulating death ligands; Secreting IFN-γ for activating other immune cells	Antigen-specific phagocytosis and antigen-independent phagocytosis; Antigen presentation; Killing achieved by inducing other immune cells
Source	Peripheral blood mononuclear cell; Hematopoietic stem/progenitor cell; Induced pluripotent stem cells	Peripheral blood mononuclear cell; Umbilical cord blood NK cells; NK cell lines (e.g. NK-92); Induced pluripotent stem cells; Embryonic stem cell	Peripheral blood mononuclear cell; Induced pluripotent stem cells
Advantages	High cytotoxicity; Adequate number of circulating lymphocytes; Sufficient exploration in CAR development and delivery; Multiple FDA-approved products;	Dual cytotoxicity: CAR-dependent and innate Low levels of CRS and ICANS; Reduced on-target/off-tumor toxicity; Richer accessible source for generation; Low risk of GvHD and more likely off-the-shelf	Innate advantages against solid tumors: capacity of powerful infiltration, remodeling the immunosuppressive TME; Antigen presentation and Immunomodulatory functions that may amplify broader immune responses; Low risk of GvHD;
Disadvantages	Adverse effects: CRS, ICANS; On-target/Off-tumor toxicity; Limited efficiency in solid tummors; T cell exhaustion; Risk of GvHD and more difficulties in transforming off-the-shelf products	Lower number of circulating CAR-NK cells; Low persistence and requires repeat dosing; More sensitive to freezing and thawing	CRS; Difficulty in transfection; Risk of conversion to a tumor-promoting phenotype; Research is in early stages with limited clinical data
Applicable situations	$CD19^+$/$BCMA^+$ hematologic malignancies	Hematologic malignancies/solid tumors, especially when rapid treatment is required or when autologous T cells are unavailable/inadequate	Solid tumors, especially in the presence of abundant tumor-associated macrophages/with significant immunosuppressive microenvironment
Reasons for applicable situations	1. Hematologic malignancies have homogeneous antigenic expression and uncomplicated TME 2. Proven products with FDA approval and clear efficacy	1. Fast and cost-effective production 2. "Off-the-shelf"product advantage as an alternative therapy for patients with limited access to T cells	1. Remodeling of immunosuppressive TME to enhance immune cell infiltration and activation 2. Presentation of antigens to amplify the immune response
CAR Products	CD7 CAR-T cell (NCT05454241) CD70 CAR-T cell (NCT05947487) GD2 CAR T cell (NCT05298995) CEA CAR-T cell (NCT06126406) ThisCART19 A (CD19 allogeneic CAR-T cell) (NCT05691153) VEGFR1/PD-L1 CAR-T cell (NCT05477927)	CD33/FLT3 CAR-NK cell (NCT06325748) NKG2D CAR-NK cell (NCT05776355) CD19 CAR-NK cell (NCT05645601) CLDN6/GPC3/Mesothelin/AXL-CAR-NK cell (NCT05410717) Universal CD19 CAR-NK cell (NCT05654038)	HER2 CAR-Macrophage (NCT06224738)
Clinical application	Remarkable success in the field of hematological malignancies; Challenges in the field of solid tumors and needs for further development	Fewer clinical trials than CAR-T cell therapy; Potential to overcome challenges of solid tumor treatment in combination with other immunotherapies	Some early clinical trials demonstrated encouraging results; Potential candidates to overcome solid tumor challenges
References	[17, 31, 302, 364]	[46, 365–367]	[365, 368, 369]

CRS cytokine release syndrome; *ICANS* immune effector cell-associated neurotoxicity syndrome; *GvHD* graft-versus-host disease; *TME* tumor microenvironment

NKT cells co-express TCR and NK cell markers (CD161) and could recognize lipid antigens via CD1d molecules [67]. CAR-NKT cells with dual antigen recognition mediated by CAR and CD1 d molecules could rapidly secrete cytokines such as IFN-γ and IL-4 upon stimulation [68], which mobilize and activate immune cells and inhibit TAMs, thus modulating the TME functionality [69]. Rotolo et al. have demonstrated that when targeting CD19, CAR-NKT cells demonstrate enhanced lymphoma eradication efficacy compared to CAR-T cells both in vivo and *in vitro* [70]. Moreover, CAR-NKT could traverse the blood–brain barrier, facilitating the clearance of cerebral lymphomas—a feat unattainable with CAR-T cell therapy [70]. The in vivo investigation of CAR-NKT cells has substantiated their proficiency in eradicating M2 macrophages that express CD1 d molecules, while simultaneously instigating epitope spreading to potentiate tumor suppression [71]. In hematological malignancies, CD33-specific allogeneic CAR-NKT cells displayed superior tumor eradication efficacy relative to CAR-T cells, including elimination of CD33-negative/low-expressing leukemia stem and progenitor cells – a capability not documented in CAR33-T cell interventions [72]. Additionally, NKT cells exhibit minimal propensity to induce GvHD, and even attenuate GvHD, which potentially through the secretion of IL-4 to modulate post-transplant immune responses [73], thereby presenting a more favorable safety profile. CRISPR/Cas9-mediated disruption of HLA class I/II complexes generated universal CAR-NKT cells that showed superior resistance to allogeneic immune rejection while maintaining enhanced antitumor efficacy compared to conventional CAR-T cells and peripheral blood mononuclear cell (PBMC)-derived NKT cell products [74].

γδ T cells harbor γδ TCRs that mediate direct recognition of tumor-associated antigens (TAAs), including phosphoantigens and major histocompatibility complex class I-related chain molecules A/B, while exhibiting innate cancer-fighting effector through cytokine secretion and cytotoxicity [75]. The cytotoxic activity of γδ T cells is independent of MHC molecular recognition. It is mediated through potent multifaceted mechanisms, including the secretion of IFN-γ, the release of perforin and granzyme, as well as the activation of TRAIL and Fas/FasL pathways [76, 77]. γδ T cells possess an enhanced homing proficiency compared to αβ T cells and are predominantly localized in epithelial tissues, such as the skin and intestinal mucosa [78], thus inherently providing a strategic advantage in combating epithelial-originated neoplasms, including gastric and pulmonary carcinomas. Rscher et al. pioneered the engineering of CAR-γδ T cells using retroviral vectors, substantiating their ability to effectively eliminate lymphoma cells *in vitro* [79]. Subsequent developed advancements, including transposon systems and mRNA electroporation [80], have further enhanced their oncolytic potency and targeting precision in animal models. Notably, γδ T cells don't elicit GvHD, and CAR-γδ T cells have manifested a promising safety profile in preclinical studies, underscoring their potential as a next-generation allogeneic CAR-T therapy [81, 82].

MAIT cells function as innate immune effector cells, recognize microbial metabolites presented by MR1 molecules through TCR-mediated interactions [83], and are typically enriched in mucosal tissues [84]. The metabolite-sensing capability of MAIT cells, which enables the detection of abnormal metabolites within TME, provides a distinctive targeting edge for the engineering of CAR constructs. In addition, the localization of MAIT cells in the mucosal barrier confers a more natural penetration advantage of CAR-MAIT in treating solid tumors. Nevertheless, the restricted extracellular expression of MR1 and the diminished abundance of MAIT cells within tumor tissues pose formidable obstacles for the clinical translation of CAR-MAIT therapy [85]. Furthermore, beyond their antitumor functions, MAIT cells exhibit oncogenic potential in certain malignancies [86, 87], plausibly inducing T-cell exhaustion through CTLA-4/PD-1/TIM-3 pathways or recruiting immunosuppressive cells via IL-8 secretion. Thus, research on CAR-MAIT cells is still in its early stages, but their contribution to mucosal immunity and antimicrobial defense suggests therapeutic promise for treating infection-associated cancers or solid tumors [88–90].

Collectively, CAR-unconventional T cells exhibit reduced classical MHC dependency compared to CAR-T/NK/M cells, rendering them more promising candidates for the development of allogeneic universal CAR cell therapies [66]. In addition, CAR-unconventional T cells are more resistant to the immunosuppressive TME while maintaining natural killing abilities. Collectively, CAR-unconventional T cells present highly promising therapeutic potential. Nevertheless, these unconventional T cells confront significant challenges, including limited source and difficulty in vitro expansion, which impede their clinical translation [91]. For instance, NKT cells constitute approximately 5% of peripheral blood, while γδ T cells account for less than 1% of PBMC [92], and both are challenging to expand in vitro to achieve therapeutically effective quantities. Several strategies have aimed to differentiate genetically engineered hematopoietic stem and progenitor cells into NKT cells for in vitro expansion, bolstering their feasibility for clinical translation potential [93, 94].

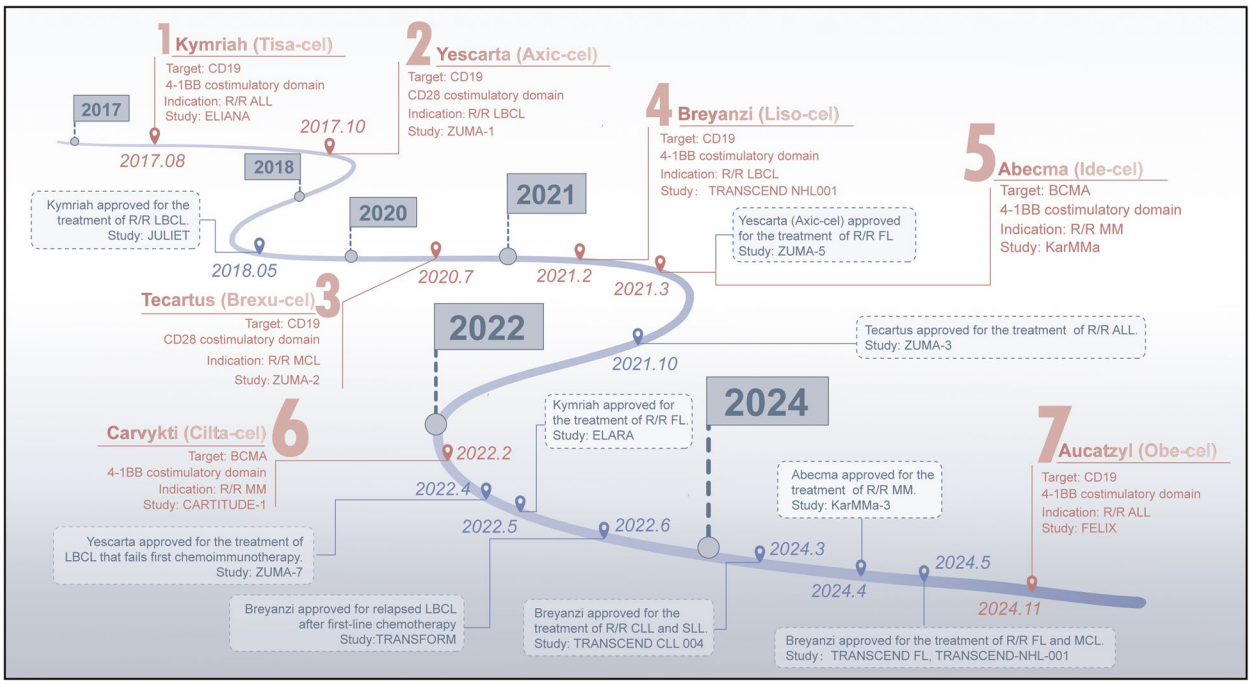


Fig. 4 FDA-approved CAR-T products

Preclinical and clinical applications of gene-edited immune cells

CAR-T cell

Since 2017, seven CAR-T cell products have been approved by the U.S. Food and Drug Administration (FDA), including 5 targeting CD19 and 2 targeting BCMA (Fig. 4). The seventh FDA-approved CD19-targeted CAR-T cell product, Aucatzyl (obe-cel), employs a rapid antigen-binding dissociation mechanism to minimize T-cell hyperactivation, thereby significantly reducing the incidence of grade ≥3 CRS (2.4%) and immune effector cell-associated neurotoxicity syndrome (ICANS, 7.1%) [95]. Notably, Aucatzyl ushered in a new era of enhanced CAR-T cell therapy risk control by integrating mechanistic innovation with rigorous clinical validation, establishing it as the first FDA-approved CAR-T cell therapy to achieve exemption from the Risk Evaluation and Mitigation Strategy [96]. This regulatory milestone reflects a paradigm shift in cellular immunotherapy and demonstrates that engineered T-cell therapies concurrently optimize safety thresholds and clinical accessibility. Recent studies have compared the efficacy of BCMA-targeted CAR-T cells (cilta-cel/ide-cel) with standard therapy in patients with relapsed/refractory (R/R) multiple myeloma. It was demonstrated that the infusion of cilta-cel had a higher overall response (84.6%, n = 208), complete response (CR) (73.1%, n = 208), negative frequency of minimal residual disease (MRD) (60.6%, n = 208), and a lower risk of disease progression (progression-free survival at 12 months: 75.9%, n = 208) [97]. Meanwhile, the early use of cilta-cel may reduce CAR-T cell-induced adverse events [97]. Another study, evaluating the efficacy of ide-cel in 254 patients with R/R multiple myeloma, reported that 71% of patients in the ide-cel group responded, with a CR rate of 39%, and substantially prolonged the median progression-free survival (13.3 months) [98]. A phase II clinical trial, evaluating CAR-T cell efficacy for high-risk R/R large B-cell lymphoma (LBCL) as a part of first-line therapy, demonstrated that axi-cel, an anti-CD19 CAR-T cell product, is both safe and efficacious [99]. Axi-cel also possessed a durable clinical benefit in elderly patients (≥ 65 years) with R/R LBCL, showing manageable toxicity, and improved clinical outcomes [100]. The therapeutic potential of CAR-T cells targeting other targets is being further explored in pre-clinical and clinical trials (Table 2), such as CD7 [101], CD22 [102], GPRC5D [103], NKG2D [104], CD123 [105], SLAMF7 [106], and Siglec-6 [107].

Although CAR-T cell products for solid tumors have not been approved, numerous clinical trials are underway and show promising results, including targeting HER2 [108], IL-13Rα2 [109], GD2 [110], ROR1 [111], EGFR [112], CEA [113], mesothelin (MSLN) [114], and CD133 [115]. In the phase I clinical trial of 37 patients with gastrointestinal tumors treated with claudin18.2-specific CAR-T cells, the overall response rate was 48.6%, the

Table 2 Clinical trials of CAR-T cells

NCT Number	Target	Clinical Data	Results	Study Status	Conditions	Phases
NCT06326463	CD70	R/R $CD70^+$ blood cancer patients (≤ 21 years) get CD70-CAR T cells post-lymphodepletion to test safety, dose, and antileukemic effect	None	RECRUITING	ALL, Childhood | AML, Childhood | Lymphoma | MDS	I
NCT06408194	CD22	Children/young adults with R/R BCL receive CD22 CAR-T cells administered 28 to 42 days after an infusion of tisagenlecleucel, aiming to determine the safety, feasibility, and the MTD/RP2D of the treatment, as well as to assess its clinical activity and the rate of ongoing B cell aplasia	None	RECRUITING	Leukemia | ALL	I
NCT06393335	CD19	R/R $CD19^+$ B-cell malignancy patients (≤ 18 years) receive metabolically armed CD19 CAR-T cells to test safety, efficacy, PK/PD, and tumor remission post-infusion	None	RECRUITING	ALL | NHL	EARLY_I
NCT06462248	CD19	Pediatric and adult patients with R/R ALL or B-cell NHL receive CD19 CAR-T cells made by CliniMACS Prodigy to test safety and effectiveness, following lymphapheresis, lentiviral transduction, and conditioning chemo, with 7-day inpatient monitoring and 2 + year follow-up	None	RECRUITING	Lymphoma, Nonhodgkin | Leukemia, Lymphocytic	II
NCT06333509	GPRC5D	Adults with R/R MM or primary plasma cell leukemia receive CT071, anti-GPRC5D CAR-T cell, therapy in a Phase I/II trial: dose escalation, expansion, then Phase II at recommended dose, with 15-year follow-up	None	NOT_YET_RECRUITING	MM | Primary Plasma Cell Leukemia	I|II
NCT05995041	AML	AML patients get universal CAR-T cells targeting CLL1, CD33, CD38, or CD123 to test feasibility, safety, efficacy, plus their function and persistence	None	RECRUITING	AML	I
NCT06345027	CD70	Patients with R/R EBV-linked NHL/HL receive C7R-EBVST cells—Group A without lymphodepletion, Group B with it—to determine max safe dose, persistence, and anti-cancer impact, with 15-year follow-up	None	NOT_YET_RECRUITING	Leukemia, Myeloid, Acute | Leukemia, B-cell | Leukemia, T-Cell | Lymphoma	I
NCT05995028	CD7	$CD7^+$ hematological malignancy patients receive 4SCAR7U CAR-T cell therapy, aiming to assess the feasibility, safety, and efficacy of the treatment, as well as the function and persistence	None	RECRUITING	T-ALL | AML | NK Cell Lymphoma	I
NCT06064903	CD7	Pediatric patients with R/R T-ALL/T-LL who have failed at least one standard frontline chemotherapy or relapsed after allogeneic HSCT receive CD7-CART01 therapy, aiming to determine MTD in Phase I and assess the efficacy and safety of the treatment at the RP2D in Phase II	None	RECRUITING	T-ALL/Lymphoblastic Lymphoma	I|II
NCT06326021	CD33	Patients (21–27 total cases) with R/R AML receive optimised CD33 (FL-33) CAR T cells to assess safety (DLT within 21 days, AEs within 30 days) and efficacy, testing two doses (5 × 10^5 or 1 × 10^6 cells/kg) to find the optimal biological dose	None	RECRUITING	R/R AML	I
NCT06196255	FcRL5	R/R MM patients receive anti-FcRL5 CAR-T cells to evaluate efficacy and safety, with leukapheresis, lymphodepletion (fludarabine + cyclophosphamide), and DLT/MTD/RP2D assessment post-infusion	None	RECRUITING	MM	I|II

Table 2 (continued)

NCT Number	Target	Clinical Data	Results	Study Status	Conditions	Phases
NCT05672147	CD33	Patients with R/R CD33$^+$ AML receive donor-derived CD33-CAR T cells after lymphodepletion in a dose-escalation study to test anti-tumor activity and safety, while assessing CAR T cell persistence, hematopoiesis impact, PFS, OS, and CD33 expression changes	None	RECRUITING	AML | Recurrent Adult AML | Refractory AML | Secondary AML	I
NCT06208735	CD22	Patients with R/R B-cell malignancies receive autologous CLIC-2201, CD22 CAR-T cells, to assess safety, tolerability, and MTD using a 3 + 3 dose-escalation design, following leukapheresis and lymphodepletion (cyclophosphamide + fludarabine), with monitoring up to 1 year for anti-tumor activity, pharmacokinetic, and immune responses	None	NOT_YET_RECRUITING	BCL | NHL | B-cell Acute Lymphoblastic Leukemia | DLBCL | High-grade B-cell Lymphoma | Primary Mediastinal Large B-cell Lymphoma | Mantle Cell Lymphoma	I
NCT06213636	CD19/CD22	Patients with R/R B-cell malignancies receive CD19/CD22 CAR-T cells in a Phase I dose-escalation trial to assess safety, tolerability, pharmacokinetics, and initial efficacy, aiming to determine the optimal dose and reinfusion schedule for Phase II, with preconditioning chemo and close post-infusion monitoring	None	RECRUITING	ALL, Adult B-Cell | ALL, in Relapse | NHL, B-cell | DLBCL | Central Nervous System Lymphoma | Lymphoma, Follicular | Mantle Cell Lymphoma	I|II
NCT05783089	Mesothelin	Patients with mesothelin$^+$ R/R cancers receive anti-mesothelin CAR-T cells to test safety, tolerability, and initial efficacy, with dose escalation and expansion across three cohorts exploring chemo regimens, administration modes (IV vs. local), and checkpoint inhibitor combos	None	NOT_YET_RECRUITING	Mesothelin-positive Advanced Malignant Solid Tumors	I
NCT05415475	CEA	CEA$^+$ advanced tumor patients get optimized anti-CEA CAR-T cells to test safety, efficacy, and find the best dose and infusion plan.	None	RECRUITING	Colorectal Cancer | Esophageal Cancer | Stomach Cancer | Pancreatic Cancer | Metastatic Tumor | Recurrent Cancer	I
NCT06248697	MSLN	Patients with solid tumors receive autologous MSLN-targeted CAR T cells secreting PD-1 and CTLA-4 nanobodies (αPD1/CTLA-4-MSLN-CAR T cells), aiming to evaluate the safety and tolerability of the treatment	None	RECRUITING	Solid Tumor	EARLY_I
NCT06612645	B7H3-IL7Rα	Kids with recurrent/unresponsive solid tumors after standard care get CAR T-cells engineered with IL-7Ra signaling to target B7H3 to test safety and early efficacy	None	NOT_YET_RECRUITING	Pediatric Cancers | Solid Tumor Pediatric	I
NCT04981691	Mesothelin	Patients with mesothelin$^+$ advanced solid tumors failing first/second-line therapy receive mRNA-engineered anti-MESO CAR-T cells to assess safety, efficacy, and pharmacokinetics, using a 3 +3 dose-escalation design with 1 × 10^9/3 × 10^9 doses, infused thrice weekly for two weeks after lymphodepletion	None	UNKNOWN	Refractory Malignant Solid Neoplasm	I
NCT06010862	CEA	Patients (about 12 per arm) with CEA$^+$ advanced/metastatic solid tumors receive CAR-T cells in a Phase I study to test safety, tolerability, and determine MTD and Phase II recommended dose, split into two arms (intravenous and intraperitoneal), each with 4 dose groups in a 3 +3 design	None	RECRUITING	Gastric Cancer | Colon Cancer | Pancreas Cancer | Esophagus Cancer | Cholangiocarcinoma | Lung Cancer | Breast Cancer	I

Table 2 (continued)

NCT Number	Target	Clinical Data	Results	Study Status	Conditions	Phases
NCT05472857	CLDN18.2	CLDN18.2-positive advanced solid tumor patients receive autologous anti-claudin18.2 CAR-T cells (IMC002) to assess safety and efficacy, involving leukapheresis, preconditioning, infusion, and long-term gene safety follow-up	None	RECRUITING	Gastric Cancer | Pancreatic Cancer | Advanced Ovarian Carcinoma | Gastroesophageal Junction Adenocarcinoma	I
NCT06590246	GPC3	$GPC3^{+}$ advanced/recurrent HCC adult patients who failed or can't tolerate ≥ 2 prior therapies receive C-CAR031, armored anti-GPC3 CAR-T cells to assess safety, tolerability, anti-tumor activity, PK, PD, and immunogenicity, determining RDE and RP2D	None	NOT_YET_RECRUITING	Hepatocellular Carcinoma	I|II

R/R refractory/relapsed; *PK* pharmacokinetic; *PD* pharmacodynamics; *ALL* acute lymphoblastic leukemia; *AML* acute myeloid leukemia; *MDS* myelodysplastic syndromes; *NHL* non-hodgkin's lymphoma; *HL* Hodgkin's lymphoma; *EBVST cells* EBV-specific T cells; *HSCT* hematopoietic stem cell transplantation; *MTD* maximum tolerated dose; *RP2D* recommended phase 2 dose; *DLT* dose limited toxicity; *AE* adverse events; *MM* multiple myeloma; *PFS* progression free survival; *OS* overall survival; *T-ALL* T-cell acute lymphoblastic leukemia; *BCL* B-cell lymphoma; *DLBCL* diffuse large B cell lymphoma

disease control rate was 73.0%, and a 6-month duration response rate was 44.8%, with tolerable adverse events [116]. Similarly, a phase I/II clinical trial of 27 children with R/R neuroblastoma treated with GD2-targeted CAR-T cells showed a higher response rate and improved 3-year overall survival [110]. Vitanza et al. reported repeatedly dosed intracranial B7-H3 CAR-T cells in three children with R/R diffuse intrinsic pontine glioma (DIPG) [117]. Among them, a patient demonstrated sustained clinical and radiographic improvement through 12 months, with evidence of local immune activation [117], suggesting the feasibility and preliminary tolerability of repeated administration. Frustratingly, most research on CAR-T cells in solid tumors still lacks adequate overall response rates and possesses serious toxicities. With ongoing clinical trials exploring novel targets, CAR-T cell therapy is poised to make further breakthroughs in the treatment paradigms for solid tumors.

CAR-NK cell

Clinical trials of CAR-NK cells have shown encouraging results. Table 3A summarizes representative CAR-NK clinical trials. IL-15 armored anti-CD19 CAR-NK cells with inducible caspase-9 safety switch had remarkable and durable cytostatic effects on cancer and tolerable adverse events in treating patients with R/R CD19-positive lymphoma [118]. Among 11 patients, objective responses occurred in 8 of them, including 7 patients who achieved CR [118]. In a recent I/II R/R B-lymphoid malignancies trial, similar IL-15 CAR-NK cells endowed NK cells with comparable efficacy to autologous anti-CD19 CAR-T cells, and exhibited lower ICANS [119]. Anti-CD70 CAR-NK cells secreting IL-15 against CD19-negative B cell lymphomas were constructed in a preclinical mouse model of a xenograft lymphoma line. Repetitive administration of the CAR-NK cells induced sustained tumor remission and enabled long-term survival of CAR-NK cells in mice [120]. Thus, pre-clinical trials of CAR-NK cells have illuminated a ray of hope in treating various solid tumors [121, 122].

CAR-M cell

Compared to other gene-edited immune cells, research in CAR-M cells is still in its infancy (Table 3B). Preclinical trials have developed CAR-M cells for solid cancer treatment, such as targeting human epidermal growth factor receptor 2 (HER2) [123–125], CD133 [126], mesothelin [127], GD2 [128], glypican-3 (GPC3) [129], anaplastic lymphoma kinase (ALK) [130], mucin 1 (MUC1), and CD19 [127]. Zhang et al. constructed a CAR-147-M targeting HER2, with a CD147 intracellular signal transduction domain, dramatically upregulated multiple MMP expression and promoted the degradation of tumor mechanical barrier, thereby suppressing tumor growth in vivo [60]. Furthermore, levels of inflammatory cytokines (IL-1, TNFα), paramount mediators of CRS, were reduced in mice treated with CAR-M cell therapy, whereas levels of anti-tumor factors (IL12, IFNγ levels) increased [60]. iPSC-derived CAR-M cells could also expand in vivo and maintain their anti-tumor efficacy [127]. The HER2-targeted CAR-M cell therapy, CT-0508, has reported preliminary outcomes from a Phase I clinical trial in HER2-overexpressing tumors, predominantly breast and esophageal cancers, demonstrating favorable tolerability, early antitumor activity, and remodeling of the intratumoral immune microenvironment [131]. Nevertheless, clinical trials of CAR-M cells have not reported final results [59].

CAR-Unconventional T cells

Up to now, clinical studies of CAR-NKT cells remain limited, with only five trials registered on ClinicalTrials.gov (Table 3C). A phase I clinical trial evaluating CAR-NKT cells co-expressing GD2 and IL-15 reported an ORR of 25% (3/12) and showed an enhanced safety profile in pediatric patients with neuroblastoma [132]. CAR-γδ T cells manifest a relatively advanced status in the research progression of unconventional T cells, particularly in hematological oncology, where early encouraging signals have been detected (Table 3D). A subset of clinical trials has advanced to phase I/II, revealing superior safety profiles and significant objective tumor regression. ADI-001, an allogeneic CAR-γδ T cell therapy targeting CD20, was evaluated in patients with relapsed/refractory B-cell lymphoma [133]. The Phase I clinical trial revealed an ORR of 67% (4/6) and a CR of 67% (4/6), while exhibiting a favorable safety profile. Allogeneic B7H3-targeted CAR-γδT cells demonstrated favorable tolerability in a clinical trial involving 7 patients with high-grade recurrent GBM, with no instances of grade 3 or higher CRS, ICANS, or GvHD, achieving an objective response rate (ORR) of 42.9% (3/7). Despite the considerable anticipation surrounding CAR-MAIT cell application in mucosa-associated solid tumors (such as colorectal, hepatocellular, and lung cancers), CAR-MAIT cells are still confined to the preclinical phase, with no research having progressed to clinical trials to date. The advancement of CAR-MAIT technology depends on additional experimental substantiation of its safety and practicability.

New challenges for immune cell therapy: multi-antigen targets gene editing

Challenges in target selection

Extending CAR immune cell therapy, the frontrunner in hematological malignancies, to the vast majority of tumors (solid tumors) represents a significant challenge

Table 3 Clinical trials of CAR-immune cells beyond CAR-T cells

Type	NCT Number	Target	Clinical Data	Results	Study Status	Conditions	Phases
A. CAR-NK cell	NCT05570188	CD19	Patients with B cell hematologic malignancies expressing CD19 receive anti-CD19 universal CAR-NK cells 1–7 days post-HSCT, aiming to evaluate the efficacy and safety in promoting engraftment and disease control, while monitoring for potential adverse reactions over 2 years	None	WITHDRAWN	BCL	I|II
	NCT05645601	CD19	Patients with R/R B cell hematologic malignancies receive JD010 (allogenic CD19-targeted CAR-NK cells), aiming to investigate the safety and efficacy of the treatment	None	RECRUITING	Adult R/R B-cell Hematologic Malignancies	I
	NCT04264078	CD7	Patients with $CD7^+$ R/R T/NK-cell hematologic malignancies receive allogeneic anti-CD7 universal CAR-T cells, aiming to provide an accessible and affordable treatment option	None	UNKNOWN	T-cell Leukemia | T-cell Lymphoma	EARLY_I
	NCT05739227	CD19	Patients with R/R B-cell hematologic malignancies receive allogenic CD19 CAR-NK cells after lymphodepleting therapy with fludarabine, cyclophosphamide, and etoposide, aiming to evaluate the efficacy and safety of the treatment and determine the MTD and RP2D	None	RECRUITING	ALL | BCL | Chronic Lymphocytic Leukemia	EARLY_I
	NCT05110742	CD5	Patients with R/R T-cell malignancies, mantle cell lymphoma, and chronic lymphocytic leukemia receive CD5 CAR-engineered IL15-transduced cord blood-derived NK cells with lymphodepleting chemotherapy, aiming to determine safety, efficacy, and optimal dose	None	RECRUITING	Hematological Malignancy	I|II
	NCT05941156	CD56	Patients with R/R NK/T cell lymphoma/leukemia receive CD56 CAR-T cell therapy, aiming to evaluate the safety and efficacy of the treatment	None	RECRUITING	Extranodal NK T Cell Lymphoma | NK-Cell Leukemia	II
	NCT06307054	CLL-1	Patients with R/R AML receive CLL-1 CAR NK cells, aiming to explore the safety, tolerability, pharmacokinetic characteristics, and preliminary efficacy of the treatment	None	RECRUITING	Relapsed Adult AML | Refractory AML	I
	NCT06242249	BCMA	Patients with R/R MM receive BCMA CAR NK cells, aiming to evaluate the safety and determine the MTD of the treatment	None	NOT_YET_RECRUITING	MM, Refractory	I|II
	NCT05410041	CD19	Patients with R/R B-cell malignant tumors (9–21 participants) receive CD19 CAR NK cells, aiming to observe the safety and effectiveness of the treatment and preliminarily evaluate the in vivo expansion and ORR	None	UNKNOWN	ALL | Chronic Lymphocytic Leukemia | NHL	I
	NCT05247957	NKG2D	Patients with R/R AML receive NKG2D CAR-NK cells in a dose-escalation study, aiming to determine the MTD and evaluate safety and efficacy	None	TERMINATED	Safety and Efficacy	NA

Table 3 (continued)

Type	NCT Number	Target	Clinical Data	Results	Study Status	Conditions	Phases
	NCT06201247	CD123	Patients with R/R CD123$^+$ AML receive universal CD123 CAR-NK cells in a first-in-human dose-escalation study, aiming to evaluate safety, tolerability, and preliminary efficacy, with assessment of DLT, AE, disease response, and PK/PD	None	RECRUITING	Relapse | Refractory AML	EARLY_I
	NCT05563545	CD19	Patients with R/R CD19$^+$ ALL receive CAR NK-CD19 therapy in a dose-escalation study, aiming to evaluate safety, dose tolerance, pharmacokinetics, efficacy, immunogenicity, and cytokine changes related to CRS/ICANS	None	COMPLETED	ALL	I
	NCT05020678	CD19	Patients with R/R B-cell malignancies receive NKX019, CD19 CAR NK cells, in a phase 1 dose-finding and expansion study, aiming to evaluate safety, tolerability, cellular kinetics, pharmacodynamics, and anti-tumor response	None	RECRUITING	Lymphoma, Non-Hodgkin | B-cell ALL | Large B-cell Lymphoma | Mantle Cell Lymphoma | Indolent Lymphoma | Waldenstrom Macroglobulinemia | Chronic Lymphocytic Leukemia | Small Lymphocytic Lymphoma | Aggressive Lymphoma | Large-cell Lymphoma	I
	NCT05092451	CD70	Patients with R/R hematological malignancies receive CD70 CAR/IL15-transduced CB-derived NK cells following lymphodepleting chemotherapy in a phase I/II study to evaluate safety, efficacy, optimal dose, cell persistence, and immune reconstitution	None	RECRUITING	BCL | MDS | AML	I|II
	NCT05487651	CD19	Patients with R/R B-cell malignancies receive KUR-502, a CD19-targeted CAR-NKT cell therapy with IL-15, in a dose-escalation study ($1\times10\text{^}7/m^2$, $3\times10\text{^}7/m^2$, $1\times10\text{^}8/m^2$) to determine MTD, evaluate safety, and assess efficacy	None	RECRUITING	NHL, Relapsed, Adult | BCL | DLBCL | ALL, Adult B Cell | ALL, Childhood | Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma	I
	NCT05194709	5 T4	Patients with advanced solid tumors receive anti-5 T4 CAR-NK cells in a single-arm, open-label, dose-escalation study ($3.0\times10\text{^}9$ and $4.0\times10\text{^}9$ cells per administration) to evaluate safety, tolerability, initial efficacy, and pharmacokinetics	None	UNKNOWN	Advanced Solid Tumors	EARLY_I
	NCT06066424	TROP2	Patients with advanced TROP2$^+$ solid tumors receive TROP2 CAR-NK cells with IL-15 in a phase I dose-escalation and expansion study to determine safety, tolerability, MTD, RP2D, antitumor activity, cell persistence, and biomarker dynamics	None	RECRUITING	Solid Tumors	I
	NCT06454890	TROP2	Patients with R/R Trop2$^+$ NSCLC receive Trop2 CAR-NK cells combined with chemotherapy in a study to evaluate efficacy, safety, and disease control over a 3-year follow-up period	None	NOT_YET_RECRUITING	NSCLC	I|II
	NCT06503497	NKG2D	Patients with pancreatic cancer receive second-line systemic chemotherapy followed by NKG2D CAR-NK cell therapy in a dose-escalation study to evaluate safety and anti-tumor efficacy	None	NOT_YET_RECRUITING	Pancreatic Cancer Non-resectable	EARLY_I

Table 3 (continued)

Type	NCT Number	Target	Clinical Data	Results	Study Status	Conditions	Phases
	NCT06478459	NKG2D	Patients with locally advanced pancreatic cancer receive intratumoral NKG2D CAR-NK cell injection guided by endoscopic ultrasound in a dose-escalation study to evaluate safety and anti-tumor efficacy	None	NOT_YET_RECRUITING	Pancreatic Cancer Non-resectable	EARLY_I
B. CAR-Macrophage	NCT04660929	HER2	Patients with HER2-overexpressing solid tumors receive HER2 CAR macrophages, CT-0508, in a first-in-human study to evaluate safety and efficacy, including intraperitoneal, radiolabeled, and pembrolizumab combination substudies	None	ACTIVE_NOT_RECRUITING	HER2-positive | Adenocarcinoma | Bile Duct Cancer | Biliary Tract Cancer | Bladder Cancer | Breast Cancer | Breast Neoplasm | Ductal Carcinoma | Hepatocellular Cancer | Lung Cancer | Non-Small-Cell Carcinoma | Ovarian Epithelial Cancer	I
	NCT06224738	HER2	Patients with $HER2^+$ advanced gastric cancer and peritoneal metastases receive HER2 CAR macrophage therapy to evaluate safety and efficacy, using adenovirally transduced autologous macrophages for systemic anti-tumor effects	None	NOT_YET_RECRUITING	Gastric Cancer	EARLY_I
C. CAR-NKT cell	NCT06394622	CD70	Patients with $CD70^+$ advanced solid tumors receive CGC729, a CD70 CAR-NKT cell therapy, in a phase I dose-expansion study (6–12 participants) following lymphodepletion with fludarabine and cyclophosphamide to evaluate efficacy, safety, and PK	None	RECRUITING	Solid Tumor	I
	NCT06728189	CD70	Patients with $CD70^+$ advanced solid tumors receive CGC738, a CD70-targeted CAR-NKT cell therapy, following lymphodepletion with fludarabine and cyclophosphamide, aiming to determine MTD and evaluate efficacy, safety, and PK	None	RECRUITING	Advanced Malignant Solid Tumors	I
	NCT06182735	CD70	Patients with relapsed/metastatic advanced RCC receive CD70 CAR-NKT cell therapy, CGC729, in a phase I dose-escalation study (3 + 3 design) to determine MTD based on DLTs and evaluate safety, tolerability, and efficacy	None	RECRUITING	Renal Cell Carcinoma	I
	NCT03294954	GD2	Patients with refractory neuroblastoma receive GD2 CAR-NKT cell therapy combined with IL-15 and CD28, along with ETANercept (a TNF inhibitor), in a dose-escalation study to evaluate safety, efficacy, persistence, and tumor response	None	RECRUITING	Neuroblastoma	I
	NCT03774654	CD19	Patients with R/R $CD19^+$ lymphoma/leukemia receive ANCHOR cells, a gene-modified NKT cell therapy combining anti-CD19 CAR, CD28, and IL-15 to evaluate safety, persistence, side effects, and potential efficacy	None	RECRUITING	Refractory B-Cell Non-Hodgkin Lymphoma | Refractory B-Cell Small Lymphocytic Lymphoma | Relapsed Adult ALL | Relapsed Chronic Lymphocytic Leukaemia | Relapsed NHL	I
D. CAR-γδ T cell	NCT04702841	CD7	Patients with R/R $CD7^+$ T-cell malignancies receive CAR-γδ T cell therapy, aiming to evaluate efficacy in a clinical study	None	UNKNOWN	Malignant Tumors	EARLY_I

Table 3 (continued)

Type	NCT Number	Target	Clinical Data	Results	Study Status	Conditions	Phases
	NCT05302037	NKG2DL	Patients with advanced cancers receive CTM-N2D, allogeneic NKG2DL CAR-γδ T cell therapy, in a phase I dose-escalation (1 × 10^7—1 × 10^9 cells/infusion) and optimal dose confirmation study, evaluating safety, tolerability, and tumor response	None	RECRUITING	Malignancy | Refractory Cancer Relapsed Cancer	I
	NCT06106893	CD19	Patients with active SLE receive CD19 universal CAR-γδT cell therapy, aiming to evaluate safety, efficacy, pharmacokinetics, and pharmacodynamics with a 2-year follow-up	None	RECRUITING	Systemic Lupus Erythematosus	I|II
	NCT04735471	CD20	Patients with R/R B cell malignancies receive ADI-001, allogeneic CAR-γδ T cell therapy, in a 3 + 3 dose escalation study, aiming to evaluate safety, efficacy, and determine MTD/MAD, with pharmacokinetic and pharmacodynamic assessments	None	ACTIVE_NOT_RECRUITING	Follicular Lymphoma | Mantle-Cell Lymphoma | Marginal Zone Lymphoma | Primary Mediastinal B-cell Lymphoma | Diffuse Large BCL | NHL	I
	NCT06480565	CD70	Patients with R/R ccRCC receive ADI-270, CD70 CAR-γδ T cells, in a phase 1/2 multicenter, open-label, dose escalation, and dose expansion study	None	RECRUITING	Clear Cell Renal Cell Carcinoma	I|II
	NCT04107142	NKG2DL	Patients with R/R solid tumors receive haploidentical/allogeneic NKG2DL CAR-γδ T cells (3 × 10^8—3 × 10^9 cells), aiming to evaluate the safety, tolerability, and feasibility of the treatment	None	UNKNOWN	Colorectal Cancer | Triple Negative Breast Cancer | Sarcoma | Nasopharyngeal Carcinoma | Prostate Cancer | Gastric Cancer	I
	NCT06375993	CD20	Patients with autoimmune disease receive ADI-001, allogeneic CD20 CAR-γδ T cell therapy, aiming to evaluate the safety and efficacy of the treatment	None	RECRUITING	Lupus Nephritis | Autoimmune Diseases | Systemic Sclerosis | Systemic Lupus Erythematosus | ANCA-Associated Vasculitis | Idiopathic Inflammatory Myopathies | Stiff Person Syndrome	I

HSCT hematopoietic stem cell transplantation; *BCL* B-cell lymphoma; *R/R* refractory/relapsed; *ALL* acute lymphoblastic leukemia; *MTD* maximum tolerated dose; *RP2D* recommended phase 2 dose; *CLL-1* C-type lectin-like molecule 1; *AML* acute myeloid leukemia; *MM* multiple myeloma; *ORR* overall response rate; *NHL* non-Hodgkin's lymphoma; *DLT* dose limited toxicity; *AE* adverse events; *PK* pharmacokinetics; *PD* pharmacodynamics; *CRS* cytokine release syndrome; *ICANS* immune effector cell-associated neurotoxicity syndrome; *MAD* maximum administered dose; *MDS* myelodysplastic syndromes; *T-ALL* T-cell acute lymphoblastic leukemia; *DLBCL* diffuse large B cell lymphoma; *SLE* systemic lupus erythematosus; *ccRCC* clear cell renal cell carcinoma

for immunotherapy. A primary factor contributing to the difficulty of treating solid tumors with conventional CAR-T therapies (autologous, single-target) is the restricted and inconsistent expression of tumor-specific antigens (TSAs) [134, 135]. Targets in hematological malignancies, such as CD19, are frequently characterized by highly specific expression in malignant B cells. In contrast, solid tumors predominantly express TAAs, which are also present at low levels in normal tissues [136]. The utilization of TAAs as targets for CAR-T cell therapies frequently leads to off-target toxicity. For instance, CAR-T cell targeting HER2, CEA, and MSLN has documented instances of pulmonary toxicity and, in some cases, fatal outcomes [137–139]. Moreover, the tumor heterogeneity inherent in solid tumors presents another substantial challenge for CAR target selection. Diverse tumor antigens are frequently expressed heterogeneously within solid tumors and across different patients. Consequently, single-target CAR-T cell therapy may only eliminate subpopulations of tumor cells expressing the target antigen, while residual antigen-negative or low antigen-expressing cells can persist and drive tumor progression [140]. The TME specific to solid tumors further complicates CAR-T cell therapy [141]. Physically, the TME impedes CAR-T cell infiltration into the tumor [142, 143]. Additionally, the inhibitory components within the TME can induce CAR-T cell exhaustion or functional inhibition, thereby further constraining the efficacy of CAR-T therapy [142, 144]. Notably, preclinical models could not fully replicate human tumor complexities, and this discrepancy significantly complicates clinical translation. CNS toxicity observed in GD2 CAR-T cell-treated neuroblastoma xenograft mice [145] has not been reported in clinical trials [146, 147]. In contrast, gastrointestinal toxicity associated with CLDN18.2 CAR-T cell therapy, as reported in clinical trials, was not detected in preclinical models [116]. Therefore, target selection for solid tumors necessitates a balance between efficacy and safety, driving the identification of TSA targets. The discovery of promising TSAs relies on the advancement of innovative technologies, such as robust high-throughput genome-wide CRISPR screening platforms, which may facilitate the identification of more suitable and effective antigenic targets for CAR-T cell therapy [148, 149].

Challenges of tumor recurrence

Even in the promising hematological malignancies, CAR-T cell therapies are not entirely trouble-free. The glaring problem is tumor recurrence after treatment, which represents a critical bottleneck limiting long-term efficacy. One of the important reasons for tumor recurrence lies in the antigenic loss/downregulation of tumors, a phenomenon that tumor cells have evolved a set of avoidance mechanisms to evade immune recognition in response to pressure from CAR-T cells [150, 151]. Potential mechanisms underlying this evasion include mutations in genes encoding antigens (e.g., CD19 [150, 152], BCMA [153, 154], lineage switching of tumor cells [155], and epigenetic modifications of gene expression [156], among others. In addition, tumor heterogeneity serves as a critical factor driving tumor recurrence [157, 158]. Tumor cells from antigen-negative clones are resistant to conventional single-target CAR-T cell therapies and may proliferate after treatment, thus leading to tumor recurrence. Equally important, factors intrinsic to CAR-T cells themselves, independent of target antigen expression, significantly influence therapeutic efficacy. The limited persistence of CAR-T cells in vivo, along with immunosuppressive signals from prolonged antigenic stimulation and the TME, could lead to CAR-T cell exhaustion, resulting in diminished efficacy of CAR-T cells in tumor suppression [159, 160].

Insights from previous therapeutic challenges highlight a critical realization: the long-term efficacy of single-target CAR-T therapies is inherently limited. Future CAR-T therapies must be designed to engage multiple targets. On one hand, targeting multiple tumor antigens can address the challenges posed by tumor heterogeneity and antigen loss. On the other hand, incorporating additional targets, such as co-stimulatory molecules or immune checkpoints, can enable functional signaling regulation of CAR-T cells, activating proliferative pathways or blocking inhibitory pathways. This approach can effectively mitigate cellular exhaustion and enhance the durability of the therapy.

Multi-antigen targeting cell strategies

The multi-targeting strategy represents a novel immunotherapeutic approach based on genetically engineered immune cells, aiming to enhance the durability and effectiveness of immunotherapy while avoiding tumor escape and recurrence. These engineered immune cells could target multiple tumor antigens through pooled CAR-T cells, introducing multiple scFvs and CAR constructs. Pooled combination of CAR-T cells entails mixing CAR-T cells with distinct targets. Another approach involves modifying CAR structural designs: (1) to fuse multiple scFvs in a single chimeric protein; (2) to introduce multiple CAR constructs targeting different targets in a single immune cell. The comparisons of different immunotherapy designs are summarized in Table 4.

Biological logic gates

OR-gate

OR-gate strategy could extend the CAR-T cell targeting antigen library to eradicate heterogeneous tumor

Table 4 Comparison between different immunotherapy designs

	Logic-gated CAR-immune cells			SUPRA CARs	Multi-specific antibodies	Multi-specific cell engagers
	OR-gate	AND-gate	NOT-gate			
Advantages	Expand the tumor antigen recognition spectrum; Overcome tumor heterogeneity and antigen escape; Enhance killing of heterogeneous tumor cells	Improve tumor targeting specificity; Reduce off-target toxicity; Enhance therapeutic safety; Temporal control (SynNotch CARs); Differential activation based on antigen expression levels (IF-BETTER gate)	Reduce off-target toxicity and improve safety; Expand the range of targetable tumor antigens	High flexibility to switch targets without changing cells; Highly tunable, control the reaction strength by adjusting the concentration of bridging molecules to improve safety	Longer half-life; Safe and highly modifiable; More generalized commercialized products	Small size and improved permeability; Lower risk of non-specific activation of innate immunity; Effective at low doses; Ease of large-scale production in microbial systems
Disadvantages	Increase off-target toxicity; Increase immune stress and leading to antigen escape; Increase difficulties in viral vector loading capacity and transduction efficiency due to larger CARs	Increase complexity in design and build; Require higher levels of antigen expression for activation, which may reduce effectiveness in tumors with uneven antigen distribution	Complex design; Challenge of balancing activation and suppression signals	Complex system requiring additional adapter molecules; Requires precise control of adapter concentration and time window; High clinical translational complexity	Poor tumor tissue penetration; Antibody-dependent cellular cytotoxicity; Cytokine release syndrome; Inducing anti-drug antibodies	Require continuous infusion due to short half-life; Cytokine release syndrome
Applicable situations	Hematologic malignancies; Prevention of relapse due to antigen loss after single-target CAR-T cell therapy	Solid tumors	Solid tumors, especially when targets are also expressed in normal tissues	Heterogeneous tumors may require dynamic target modulation for therapy	Hematologic malignancies	Hematologic malignancies; Solid tumors
Reasons for applicable situations	Hematologic malignancies have relatively homogeneous antigen expression, and heterogeneity is mainly manifested by antigen loss. OR-gate effectively reduces the risk of antigen escape and improves therapeutic efficacy by targeting multiple antigens simultaneously	Targets (TAAs) of solid tumors are mostly co-expressed in normal tissues and require different combinations of TAA (double recognition/multiple recognition) to improve specificity and reduce off-target toxicity	NOT-gate protects normal tissues co-expressing target antigens via iCAR	1. Modular design allows adaptation of targets to tumor heterogeneity in response to changes in tumor antigen expression 2. Dynamic regulation can be realized by changing zipFvs	1. Tumor cells in blood/bone marrow are easily accessible 2. Tumor microenvironment in hematologic malignancies is less complex	1. BiTEs are widely used in hematological malignancies and have FDA-approved products 2. Small-sized cell engagers could infiltrate solid tumors
References	[196, 339, 340, 370]	[196, 340, 370]	[196, 340]	[207, 208]	[264, 371, 372]	[264, 373, 374]

cells. Conjointly targeting CD19 and CD123 augments the possibility of eliminating subclones that may acquire selective advantages under solely targeting CD19, thus preventing the recurrence of CD19-negative tumors [161]. Of note, the simultaneous administration of two CAR-T cell lines exerts considerable immunological pressure on tumor cells, potentially leading to the concurrent escape of both targeted antigens. Pooled CAR-T cell therapy, namely concurrently administering different CAR-T products at a specific ratio [162], and cocktail therapy, which administers different CAR-T cell products sequentially at specific time intervals, both enhance tumor remission rates and reduce relapse risk [163]. The OR-gate strategy enables the engineered cells, such as tandem CAR-T cells, dual CAR-T cells, and trivalent CAR-T cells, to recognize two or more targeted antigens, and recognition of any one of these targets can result in their activation (Fig. 5A).

Dual CAR-T cells could express two independent CAR constructs, each specific for cognate antigens. Approximately 30% of patients with R/R B-ALL experience CD19-negative relapses. OR-gate dual CAR-T cells targeting CD19 and CD123, developed by Ruella et al., could prevent tumor recurrence due to antigen loss after CD19 targeting therapy [161]. Collectively, the dual CAR-T cells demonstrated superior efficacy over single antigen-targeted CAR-T cells or pooled CAR-T cells.

CAR constructs of tandem CAR-T cells contain two tandem-specific scFv structural domains. Tandem CAR-T cells demonstrate more durable activity compared to dual CAR-T cells or pooled CAR-T cells and do not exhibit increased exhaustion [23]. When simultaneously binding two antigens, tandem scFvs manifest synergistic effects, forming superior and stable immunological synapses that induce an exponentially more efficient response in tandem CAR-T cells [164]. BCMA/CD19-targeting tandem CAR-T cells for treating multiple myeloma (MM) achieved high therapeutic responsiveness while exhibiting good tolerability and safety control [165]. Tandem scFv structural domains recognizing CD19/CD20 have been designed to prevent antigen escape, which could be efficiently activated by either target antigen [166]. Likewise, OR-gate BCMA/CD319-targeted CAR-T cells for MM treatment also demonstrated superior efficacy compared to pooled CAR-T cells and dual CAR-T cells [167]. The study comparing the efficacy of tandem CD19/CD22 dual CAR-T cells and sequential CD19/CD22 CAR-T cells reported similar immune responses for both, but both were superior to single CD19 CAR-T cells [168]. Targeting CD19 and CD22 could elicit long-term MRD-negative remission in patients with R/R B-ALL [169]. Notably, the larger size of tandem CAR constructs poses a significant concern, as this could exceed the cargo capacity of viral vectors and impact transduction efficiency [170]. And incorrect pairing and hydrophobic interactions between VH and VL domains could result in scFv aggregation, potentially leading to CAR-T cell exhaustion [171, 172]. Additionally, murine-derived scFvs elevate immunogenicity risk in tandem CAR-T cells [173]. Pleasantly, developing fully human heavy chain variable antibodies and nanobodies offers promise for addressing these challenges [21, 174].

Interestingly, CAR-T cells binding three different receptors have been reported to minimize the potential for tumor antigen escape and effectively kill multiple tumor cells [175, 176]. In patients with glioblastoma (GBM), CAR-T cells targeting three antigens, including human epidermal growth factor receptor 2 (HER2), interleukin-13 receptor subunit alpha-2 (IL13Rα2), and ephrin-A2 (EphA2), have demonstrated the capacity to overcome antigenic heterogeneity, and achieved nearly complete tumor cell clearance [176]. The superior activity against GBM may be attributed to trivalent CAR-T cell-enhanced antigen-binding capacity, signaling, and immune synapse formation.

AND-gate

OR-gate could augment CAR-T cells' antigen recognition capacity, thereby arousing stronger tumor-antagonizing immunity. Nevertheless, OR-gate may lead to on-target, off-tumor toxicity. To enhance the existence and functional maintenance of genetically engineered cells in healthy tissues, AND-gate represents a seminal advance for multi-targeting strategy development [177].

(See figure on next page.)

Fig. 5 Overview of logic gates. **A**. OR gate Dual CAR (with complete co-stimulatory and activation domains per receptor), Tandem CAR and Trivalent CAR belong to the OR-gate design, where activation of CAR-immune cells can be triggered by either one of the targeted antigens. **B**. AND gate Dual CAR (with separated co-stimulatory and activation domains), On-Switch and SynNotch CAR belong to the AND gate design, requiring the simultaneous presence of two targeted antigens to activate CAR-immune cells. ON switch: The structure of CAR is divided into two parts, one carrying the intracellular structural domain of FKBP12 and the other carrying the structural domain of FRB. As an inducer, rapamycin bridges FKBP12 and FRB to assemble a functional CAR that is inactivated (OFF) in the absence of rapamycin and activated (ON) upon addition to achieve spatiotemporal regulation of toxic effects. **C**. IF-BETTER gate The presence of Tumor-Associated Antigen (TAA) 2 facilitates the activation of CAR-immune cells in the absence of TAA1. However, it is not a necessary condition for activation when TAA1 is present in sufficient amounts. **D**. NOT gate Binding of TAA2 to the CAR induces the activation of immune cells, whereas this activation does not occur in the presence of TAA1

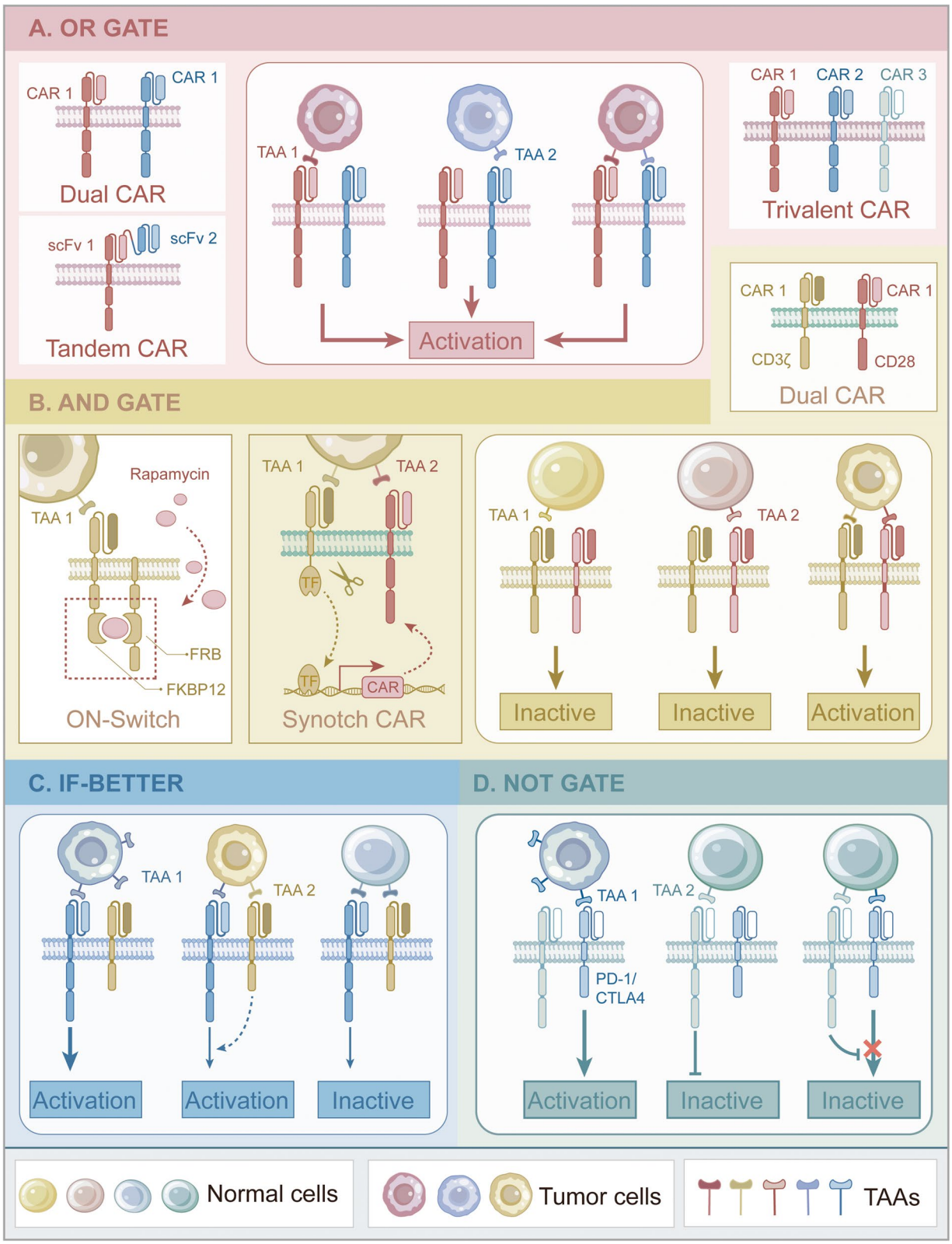


Fig. 5 (See legend on previous page.)

The ON-switch CAR incorporates a split-receptor design, where the scFvs from traditional CARs are divided into two separate parts that could assemble in a small molecule-dependent manner, effectively functioning as an ON-switch for CAR-T cell activation (Fig. 5B). This design, representing the earliest forms of AND-gate, enables precise control over the timing, spatial distribution, and activity levels of CAR-T cell activation, thereby reducing off-target risks. Wu and colleagues demonstrated the controllability and efficacy of modified rapamycin by harnessing ON-switch to form functional CD19-targeted CAR-T cells to reduce tumor load in leukemia mice [178]. The activation of AND-gate CAR design necessitates two separate target recognitions. In the AND logic gate, T-cell lysogenic cytotoxicity could be triggered only when both CARs specifically bind to the corresponding target antigen [179] (Fig. 5B). Normal tissues may express one type of tumor-associated antigen (TAA) alone at low levels, but rarely both. Thus, the combined activation signal input improves CAR-T cells' ability to discriminate between tumor and normal cells.

Similar to split ON-switch receptors, AND-gated dual CARs typically split the signaling domain, with one CAR providing a co-stimulatory signal (CD28/4-1BB) and the other providing an activating signal (CD3ζ), each targeting a different antigen. Dual CAR-T cells can be partially activated when recognizing a single target antigen with a low level of cytokine secretion. Conversely, when two target antigens are present simultaneously, dual CAR-T cells could be fully activated and exhibit anti-tumor activity. AND-gated trivalent CAR-T cells could target tumor site-specific expression patterns, such as prostate stem cell antigen (PSCA), transforming growth factor-β (TGF-β), and IL-4, and their effector functions could be confined to the pancreatic cancer site [180]. CAR-T cells targeting tumor site-specific expression patterns exhibit resilience to suppressive immune factors (TGF-β and IL-4), and this targeting approach can be extrapolated to other suppressors within TME [180]. For instance, AND-gate-regulated CAR-T cells could target suppressive factors in TME, including chemokine-receptor network, tumor vasculature [181, 182], extracellular matrix [183, 184], hypoxia [185], and immunosuppressive cytokines [186, 187], to enhance CAR-T cell survival and tumor infiltration, thereby providing a promising avenue for solid tumor treatment. Hou et al. developed bispecific CAR-T cells targeting IL-13Rα2 and TGF-β, which could counteract TGF-β-mediated immune suppression and reshape the TME, significantly driving tumor clearance in glioblastoma multiforme [187]. It was reported that IL-15-modified CAR-T cells could additionally target myeloid-derived suppressor cells to reverse their immunosuppressive effects, enhancing antitumor activity [188].

Synthetic Notch (SynNotch) receptor represents an enhanced iteration of the ON-switch, namely the IF–THEN gate. SynNotch receptor system can limit CAR expression through temporal modulation, thereby reducing non-specific attacks on healthy tissues and enhancing the safety of CAR-T cell therapy [189–191]. The specific binding of CARs targeting antigen A and SynNotch receptors could induce the release of transcriptional regulatory factors [189]. Transcription factor is translocated to the nucleus and binds to its activation sequence, which regulates transcription and expression of antigen B-specific CAR. This process results in transient expression of antigen B-specific CAR, thereby enabling CAR-T cells to express specifically only at tumor sites and ensure highly specific killing [189]. Cortese et al. describe a HER2 SynNotch-driven CEA-CAR-NK cell in which CEA-CAR expression is induced only upon amplified HER2, providing an innovative and safe strategy for treating HER2-amplified colorectal cancer (CRC) resistance [192]. In GBM, IF–THEN gate-regulated CAR-T cells achieve high specificity and more complete killing of heterogeneous tumors by employing the highly tumor-specific neoantigen EGFRvIII or the tissue-specific antigen MOG as initiating signals through a multi-targeting strategy [193]. In addition, SynNotch receptors could enhance CAR-T cell efficiency by inducing local cytokine interleukin production, thereby counteracting the immunosuppressive TME [194]. It has been shown that CAR-T cells regulated by the SynNotch system better control tumor load via circumventing the premature differentiation and depletion of CAR-T cells induced by antigen-independent tonic signaling [193, 195]. However, murine-derived SynNotch systems and synthetic transcription factors may elicit immunogenic responses, and the transgene size may pose an additional challenge in this context. Zhu et al. developed a humanized SynNotch-like system for precise regulation of gene-edited T cells by expressing customizable synthetic intramembrane proteolysis receptors (SNIPRs) [189]. Transmembrane and near-membrane structural domains of this receptor could be tuned to the sensitivity of SNIPR by altering the amino acid sequence, providing greater flexibility in customization. Interestingly, the SNIPR system could trigger recognition signals at tumor sites exclusively, which in turn drives the release and expression of ligand-dependent transcription factors. This confines CAR construction to tumor sites, reducing T-cell depletion caused by tonic signals. Additionally, SNIPRs have a compact structure and are compatible with humanized transcription factors, significantly lowering the risk of immunogenic responses.

The IF-BETTER gate could recognize both target antigens. When antigen A is present at high levels, T-cell activation does not require antigen B, as sufficient levels of antigen A alone could trigger T-cell activation [196] (Fig. 5C). Nevertheless, when the expression level of antigen A is low, antigen B contributes to reaching the activation threshold for signaling following antigen A-specific CAR-mediated recognition [196]. The regulatory logic gate implies that CAR-T cells will exhibit a preference for antigen B. Currently, the IF-BETTER gate is only applied in pre-clinical models of hematological malignancies. Through the quantitative analysis of antigen distribution and density in both acute myeloid leukemia (AML) and normal hematopoietic cells, Haubner et al. engineered a sensitivity-optimized adhesion G protein-coupled receptor E2 (ADGRE2)-CAR co-expressed with a C-type lectin domain family 12 member A (CLEC12 A)-targeted chimeric costimulatory receptor (CCR), which preferentially engages ADGRE2^{positive}/CLEC12 A^{positive} leukemic stem cells, rather than ADGRE2^{low}/CLEC12 A^{negative}, thereby minimizing off-target toxicity and effectively overcoming antigen escape mechanisms in AML [197]. Given the low expression of TSAs in solid tumors, IF-BETTER may exert far-reaching immune activity in treating solid tumors. Furthermore, the particular identification of antigen B in the IF-BETTER gate could rely on either a chimeric costimulatory receptor or a cell-surface scFv, thus circumventing the second CAR-induced, off-tumor toxicity observed in OR-gate configurations [196].

NOT-gate

NOT-gate engineered T cells could specifically recognize two target antigens (e.g., A and B). In contrast to conventional T cell activation by CAR-specific recognition, NOT-gate employs an inhibitory CAR (iCAR). The inhibitory signaling domains contained in iCARs are frequently designed to target antigen B that present solely in normal tissues to suppress cell activation (Fig. 5D). Under the regulation of NOT-gate, T cells can be activated exclusively when CAR recognizes antigen A and iCAR does not recognize antigen B. Programmed death (PD)−1/cytotoxic T-lymphocyte-associated protein (CTLA)−4-based iCAR demonstrated the superior ability to discriminate between target and off-target cells in vivo and in vitro [198]. Equivalently, Bangayan and colleagues generated iCARs with two inhibitory signaling structural domains (PD1-PD1, PD1-sialic acid binding Ig-like lectin 9, PD1-leukocyte associated immunoglobulin-like receptor 1) to regulate the delicate balance of conducting signals between CAR and iCAR, significantly improving the inhibitory efficiency of iCARs [199]. By employing iCARs to target the frequent HLA loss in tumor cells, the mechanism of tumor immune escape has been ingeniously exploited to provide a more precise and safe, yet effective strategy for CAR recognition [200–202]. Bassan et al. introduced an iCAR containing the leukocyte immunoglobulin-like receptor B1 (LIR1) inhibitory domains to target HLA-A*02 ("A2") loss of heterozygosity, thereby avoiding on-target, off-tumor toxicity in normal tissues [201]. Equivalently, Hwang et al. developed a NOT-gate-based allele-sensing CAR to target tumor cells with frequent HLA loss, and the CAR could provoke potent anti-tumor immunity in *vivo* and in *vitro* models [200]. Emerging studies are exploring potential NOT-gate-based therapeutic targets to combat tumors, emphasizing that NOT-gate could facilitate the safer and more efficacious therapeutic target development [203].

Adding the suicide switch to genetically engineered cells is a traditional strategy to improve safety [204]. The suicide switch activation leads to the complete destruction of the engineered cells, which irreversibly terminates complex therapies and renders treatments costly [205]. In contrast, the NOT-gate mechanism involving iCAR-mediated transient and reversible functional inhibition avoids these drawbacks [198]. Furthermore, after separation from the inhibitory antigen, NOT-gate CAR-T cells still maintain their reactivity to the target antigen. Therefore, NOT-gate could expand targeted tumor antigen profiles, promoting the application of tumor antigens that are highly expressed in tumor tissues but are also expressed to some extent in healthy tissues. The combined OR-NOT logical gating brings a novel approach to multi-targeting strategies. Frankel et al. introduced NOT-gating-based endomucin, a specific target for hematopoietic stem cells, to develop OR-gating-based FLT3/CD33 targeting CAR-NK cells [206]. The OR-NOT three-input logic gate CAR-NK cells can specifically target tumor cells while protecting normal hematopoietic stem cells, thereby providing a wider therapeutic window for hematological malignancy treatment.

The split, universal, and programmable (SUPRA) CAR

Adapter-mediated CAR-immune cells have the potential to overcome the limitations of tumor heterogeneity and traditional targeting paradigms and to modulate therapy in a temporal and spatial manner [207–209]. The split, universal, and programmable (SUPRA) CAR system with a modular design can theoretically target almost any antigen. THE SUPRA CAR system consists of two components [209] (Fig. 6A): (1) a universal receptor containing a leucine zip adapter (zipCAR) that connects to intracellular signaling structural units; (2) a separate switch molecule containing a homologous leucine zip with antigen-specific scFv (zipFv). SUPRA CAR-T cells containing zipCAR remain functionally inactive and can only exert anti-tumor activity when a zipFv containing

a homologous leucine zipper is exogenously added and combines with zipCAR to reconstitute a functional CAR. Based on this platform, the SUPRA CAR could switch targeting antigens by altering the exogenous switch molecule's scFv without designing a new genetically engineered cell [210]. Moreover, through modulating the zipFv, such as by increasing the concentration of switch molecules or introducing competitive binding of scFv, SUPRA CAR-T cells could regulate anti-tumor response strength. This not only broadens the spectrum of targeted antigens but also enhances the safety of genetically engineered cells for in vivo applications [210]. Designing dual or trivalent CAR systems often necessitates precisely regulating receptor expression levels and fine-tuning scFv affinities to achieve an optimal balance of signaling strength against different antigen combinations [211]. In contrast, the SUPRA CAR system modulates receptor signaling strength by altering the zipFv composition or adjusting the zipFv quantity [207].

The combination of SUPRA CAR-T cells and logic gates sheds novel inspiration on evoking robust anti-tumor efficacy and biosafety. Adding different combinations of zipFvs (α-Axl zipFv, α-Her2 zipFv, or both) to mixtures of HER2/Axl-expressing cancer cells and RR zipCAR-expressing T cells effectively activated T cells in response to any of the combination antigens [207] (Fig. 6A). SUPRA CAR-T cells incorporating NOT-gates remarkably lessen the toxicities associated with tumor-antagonizing immunity. Binding of an A-specific zipFv to the zipCAR could be prevented by administering zipFvs that specifically recognize antigens A and B, respectively, where a antigen B-specific zipFv could dimerize with an antigen A-specific zipFv [212]. The SUPRA CAR-T cells based on NOT gates can prevent the reconstruction of an antigen A-specific CAR when antigen B's safety signal is recognized, thereby circumventing off-target killing issues (Fig. 6A). Cho et al. found that the generation of an inhibitory zipCAR via the co-suppressor receptor BTLA led to a robust inhibition of IFN-γ secretion from T cells [208]. A ZipFv containing the B and T lymphocyte-associated inhibitory domain endows NOT logic function in NK cells, reducing cytotoxicity in mouse xenograft models, and establishes a bridge between CAR-immunotherapy and augmented safety [208]. Notably, the administration of SUPRA CAR requires periodic injection of zipFv protein, and the introduction of exogenous pharmaceutical agents presents several challenges, such as small molecule penetration and immunogenicity. Recently, Tousley et al. [213] put forth a novel adapter-mediated CAR, the LINK CAR, comprising fully humanized components and a modular design. The elegant LINK CAR design could address the aforementioned limitations with high efficacy while preventing off-target toxicity [213].

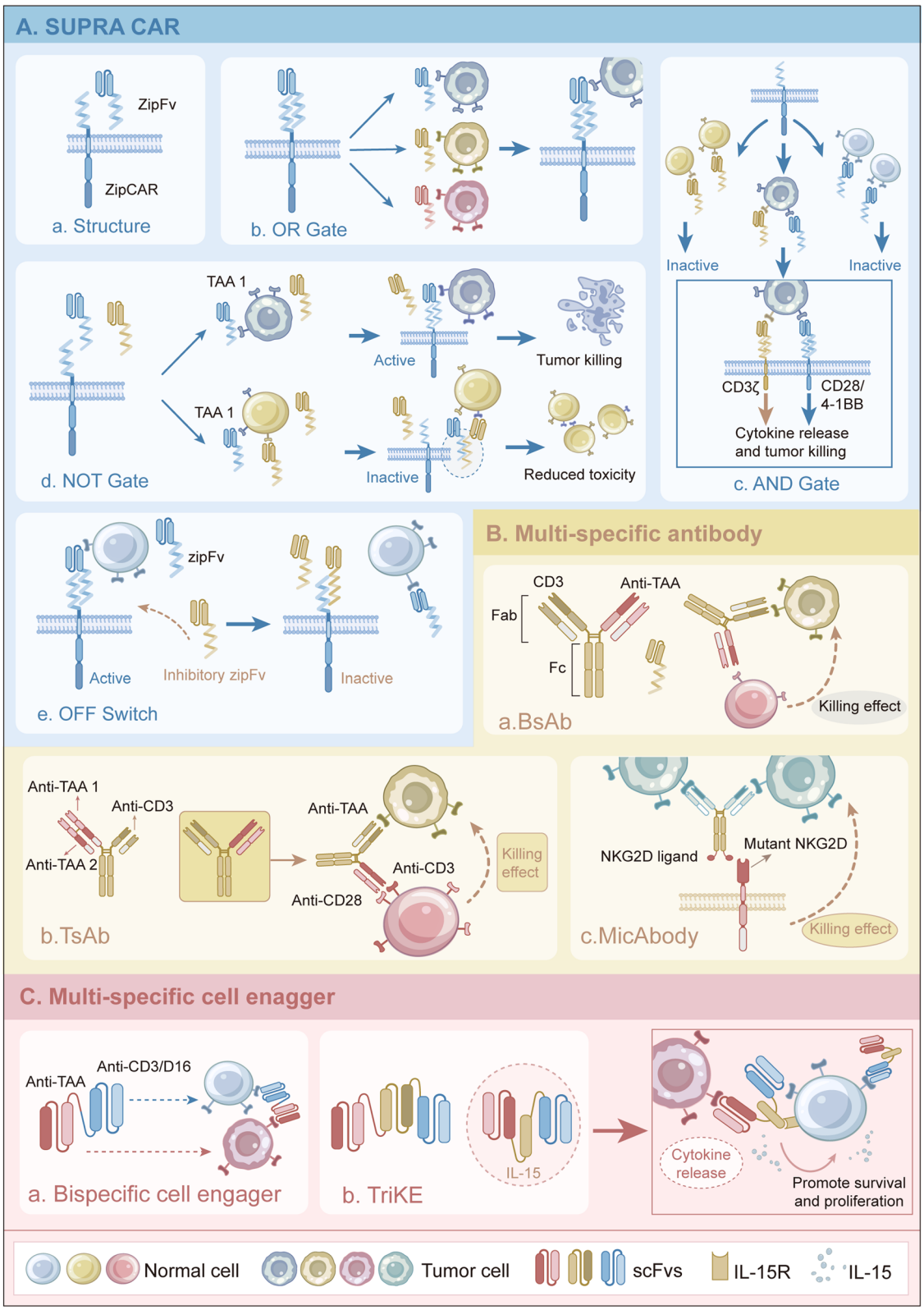
Multi-specific antibodies

BsAb

Bispecific antibody (BsAb), an engineered antibody binding to two distinct antigens or epitopes, shows broader therapeutic potential than conventional antibodies that possess a Y-shaped structure with two identical antigen-binding sites (Fig. 6B). BsAb represents an additional strategy for redirecting T cells. BsAb could bind to diverse epitopes or antigens, diverging from conventional bivalent, monospecific antibodies, which have been developed for the application of drug delivery, specific blockade, redirecting effector cells, and multi-targeting. BsAb could remodel T cells by targeting both TAAs and the CD3 complex, thereby broadening the spectrum of antigens targeted by T cells and allowing T cell-mediated cytotoxic activity through the establishment of a cytolytic synapse. Endowing BsAbs with antigenic targeting and co-stimulatory structural domains (4-1BB/CD28) agonism could transcend the limitations of liver toxicity or

(See figure on next page.)

Fig. 6 SUPRA CAR, multi-specific antibody, and multi-specific cell engager. **A**. SUPRA CAR a. Structure of SUPRA The structure of SUPRA involves a zipCAR and a zipFv, with the scFv targeting TAAs. b. SUPRA CAR based on OR gate Any zipFv with scFvs targeting different TAAs can bind to the zipCAR, mediating the activation of gene-edited immune cells. c. SUPRA CAR based on AND gate The zipFv with scFvs targeting different TAAs must simultaneously bind to the corresponding zipCAR to promote the activation of gene-edited immune cells. d. SUPRA CAR based on NOT gate Introducing zipFvs targeting two different TAAs, where TAA2 is absent on tumor cells (expressed only on normal cells), allows gene-edited immune cells to target and kill tumor cells. In normal tissues, the presence of TAA2 results in dimerization of the zipFv targeting TAA1 with the zipFv targeting TAA2, preventing the activation of gene-edited immune cells and reducing toxicity to normal tissues. e. OFF switch Introducing inhibitory zipFvs that competitively bind to the zipCAR with the zipFv targeting TAA can inhibit the activation of gene-edited immune cells. **B**. Multi-specific antibody a. BsAb The Fab segments of a BsAb target CD3 and a TAA, respectively, linking immune cells and tumor cells to promote tumor killing. b. TsAb TsAb introduces an additional anti-CD28 fragment, which, while linking immune cells, also binds to co-stimulatory domains, enhancing the oncolytic effect. c. MicAbody Based on natural killer group 2 member D (NKG2D), MicAbody is a specific type of bispecific antibody. NKG2D triggers the activation and proliferation of immune cells by recognizing and binding to activating ligands expressed on immune cells, thereby promoting the killing of target cells. **C**. Multi-specific cell engager a. Bispecific cell engager One arm of the bispecific antibody targets the CD3ε/CD16 chain to activate T/NK cells, while the other arm targets a TAA, promoting the formation of a cytolytic synapse to mediate tumor cell killing. b. TriKE TriKE contains scFvs that specifically bind to the activating receptor CD16 on NK cells and TAA, linked to the cytokine IL-15. IL-15 induces the activation and expansion of NK cells while avoiding off-target toxicity



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poor efficacy caused by co-stimulatory signaling agonism alone [214, 215]. Transformable CARs were generated by constructing bispecific antibodies (MicAbodies) to bind to the inactive NKG2D domain, the extracellular portion of the CAR, allowing for flexible targeting of CAR-T cells to bulk masses of TAAs via MicAbodies [215, 216]. Some researchers extended this platform beyond tumors, demonstrating its potential for killing HIV-infected $CD4^+$ T cells [217] (Fig. 6B). In mouse and Macaca fascicularis models, CLDN18.2 ×4-1BB-targeted BsAbs could evoke robust anti-tumor efficacy and durable immune memory responses via activating tumor-local 4-1BB signaling without inducing CRS or hepatotoxicity [215]. The combination of immune checkpoint blockade and 4-1BB agonism offered a renewed direction for enhancing immunotherapy efficacy. Specifically, tetravalent PD-L1 ×4-1BB BsAbs could convert "cold tumors" to "hot tumors", prominently reactivating exhausted T cells to exert anti-tumor activity [218]. The phase I/II clinical trial reported that CD3 ×GD2-targeted BsAb-armed T cells, in combination with IL-2 and GM-CSF, could enhance efficacy in treating neuroblastoma, exhibiting massively enhanced tumor-antagonizing activity and a favorable safety profile [219].

TsAb

Building on the BsAb foundation, tri-specific antibodies (TsAbs) further enhance the tumor-suppression capacity of immune cells by targeting multiple antigens simultaneously and introducing co-stimulatory signals [220] (Fig. 6B). The CD38 ×CD3 ×CD28-targeting TsAb in myeloma mice could induce vigorous immune responses, efficaciously facilitating T-cell activation. The co-stimulatory structural domain CD28 served as an additional target for myeloma cells, thus strengthening the specific recognition and eradication of tumor cells [220]. Additionally, tri-specific antibody construction introduces an Fc mutation for ablating FcγR binding, reducing adverse effects related to non-specific cytokine release. Inspiringly, IMT030122, a tri-specific antibody, could promote $CD8^+$ T cell activation via targeting EpCAM, 4-1BB, and CD3, executing far-reaching tumor suppression [221]. Ye and colleagues validated that tri-specific nano-antibodies targeting PD-L1, 4-1BB, and NKG2A (or TIGIT) could effectively trigger NK/T cell activation, while engaging co-stimulatory and co-inhibitory receptors to initiate innate and adaptive immunity, demonstrating greater efficiency in promoting immune cell proliferation and activation compared to BsAbs that regulate a single immune target [222]. Likewise, GNC-038, a tetra-specific antibody was demonstrated to be a highly proficient nexus, further suppressing the $CD19^+$ tumor progression [223]. A pioneering platform has recently been conceived that employs monoclonal antibodies to induce NK cells to exert ADCC function. This innovative approach, which does not entail genetic modification, allows for the production of "off-the-shelf" CAR-like NK cells and facilitates the facile targeting of various TAAs [224].

Cell engagers

Cell engagers serve as a bridge between TAAs and immune cells, facilitating the formation of a lytic immune synapse that promotes cytolytic protein secretion and enables the sequential killing of tumor cells even at low cell engager concentrations [225, 226]. Notably, the effector cell recruitment by cell engagers typically follows the targeting of TAAs, thereby ensuring tumor-specific immune cell activation. Predominantly, cell engagers are comprised of distinct scFvs, whereas those retaining the Fc domain are often engineered to abrogate its functional activity, thus mitigating the risk of off-target T cell activation or lysis and better unleashing the potential of immunotherapy [227].

Bispecific cell engagers

Bispecific T cell engagers (BiTEs) have paramount implications for battling cancer cells. Representing a class of tandem scFv BsAbs, BiTEs could specifically activate T cells by recruiting them through a binding domain targeting the CD3ε chain, and another arm targets TAAs (Fig. 6C). Given the diminutive dimensions of BiTEs, T cells and tumor cells maintain a proximate position following their conjugation via this construct, thereby facilitating the formation of a cytolytic synapse that enables the release of granzyme B or perforin from T cells [228]. Notably, this BITE-mediated formation of cytolytic synapses doesn't depend on MHC antigen presentation and TCR recognition, implying that BiTE may counteract the immune escape dilemma and reinforce CAR-T cell recognition. Furthermore, BiTEs display exceptional properties compared to conventional BsAbs, including eliciting efficacious cell killing at minimal concentrations without the necessity of prior T-cell activation. Tarlatamab (AMG757), a BiTE product targeting DLL3, remarkably retarded cancer growth for small-cell lung cancer (SCLC) treatment [229, 230], and the FDA is currently reviewing the biologics license applications submitted for this product. By employing a similar strategy to BiTEs, another study designed mesothelin-targeting CAR-T cells capable of secreting a FAP-specific T-cell engaging molecule (TEAM), which remodels the tumor stroma and mobilizes T-cell activity to enhance targeting efficacy against pancreatic ductal adenocarcinoma (PDAC) [114].

Bispecific killer cell engagers (NKCE) could recruit NK cells and target tumor sites by replacing the anti-CD3ε structural domain in a BiTE with CD16, possessing

excellent anti-tumor activity and inducing deeper cancer regression in pre-clinical studies. AFM13, a prominent representative of NKCE, could recruit NK cells to $CD30^+$ tumor regions by binding CD16 on the NK cell surface [231]. Combining AFM13 with in vitro-induced NK cell expansion may tackle the hurdles posed by tumor recognition and NK cell activation, offering a novel tactic for treating $CD30^+$ hematological malignancies [231]. An innovative alkaline phosphatase (ALP)-responsive and transformable bispecific conjugate (Supra-BiCE) has been validated to mobilize NK/T cells by self-assembling into nanoribbons and transforming into long nanoprotofibers in regions of elevated ALP expression, thereby enhancing the retention and accumulation of NK/T cells within the TME [232].

Cytotoxic cell engagers are developed by bispecific antibodies that are specific for the non-ligand-binding site of the high-affinity immunoglobulin G receptor (FcγRI). FcγRI, expressed in cytotoxic immune cells, includes monocytes, macrophages, dendritic cells, and interferon-gamma (IFN-γ)-stimulated polymorphonuclear neutrophils, and could recruit cytotoxic immune cells to the tumor site for cytotoxicity killing. In preclinical studies, targets of HER2/neu [233] and epithelial cell adhesion molecule (EpCAM) [234] boosted the anti-tumor responses and slowed tumor occurrence and progression.

Multispecific cell engagers

Multi-specific cell engagers targeting multiple TAAs significantly improve the toxicity profile of eliciting anti-cancer responses, exhibiting pleiotropic activation and synergistic effects of immune cells and achieving broader functionality compared to bispecific cell engagers. Tri-specific T cell engagers (TriTEs) integrating co-stimulatory signals into BiTEs could potentiate T cell activation and augment tumor-antagonizing responses, while introducing additional TAA targets mitigates the risk of immune escape due to antigen loss. Tapia-Galisteo and colleagues devised a TriTE for CRC treatment that contains CD3, EGFR, and EpCAM-specific recognition structural domains, which could specifically eliminate $EGFR^+/EpCAM^+$ tumor cells, whilst not exerting cytolysis on double-negative cells or inducing T-cell activation alone [235]. TriTE targeting human serum albumin (HSA), CD3, and Claudin 18.2 (CLDN18.2) manifests considerable tumor suppression of gastric and pancreatic cancers in xenograft mouse models, emphasizing that multi-targeting fueled the anti-cancer activity [236]. Endowing immune cells with resistance to inhibitory signals via TriTE could effectively circumvent the obstacles posed by immunosuppression in solid tumors [237]. The dual inhibitory immune checkpoint (ICP)-targeted nanobody-based TriTE, designed for HLA-G × PD-L1 × CD3, exhibited enhanced tumor suppression and prolonged survival in a human-derived non-small cell lung cancer (NSCLC) xenograft model utilizing immunodeficient mice [238]. Additionally, checkpoint inhibitory T cell-engaging antibody (CiTE) mitigates immune escape risk induced by PD-L1 upregulation and reduces the incidence of immune-related adverse events [237, 238]. Herrmann and colleagues have validated that αCD3 × αCD33-targeted CiTE has high selectivity for $CD33^+/PD\text{-}L1^+$ cells in vitro and in mouse models. Interestingly, CiTE did not target solely $PD\text{-}L1^+$ cells, highlighting CiTE's satisfactory safety profile in addition to markedly reinforced anti-tumor efficacy [239]. Ding et al. evaluated the functional activity of nanoantibody-based TriTEs in a xenograft mouse model, demonstrating that TriTEs possessed a superior capacity for T-cell infiltration compared to BiTEs while eliciting no toxic responses [240]. Moreover, combining multispecific T-cell engagers with oncolytic virotherapy also offers an interesting perspective for addressing the challenge of tumor heterogeneity and T-cell infiltration [241].

Various studies have endeavored to develop trispecific Killer Engagers (TriKEs) that enhance BiKEs functionality [242–244] (Fig. 6C). Cross-linking of IL-15 to an antibody manifests to significantly improve NK cell survival *in vivo* [245]. Felices et al. designed a novel tri-specific killer engager comprising scFvs that specifically bind to CD19 and the NK cell activation receptor CD16, with both scFvs linked to IL-15 [246]. IL-15 TriKEs could restore NK cell functional deficits, such as impaired cytokine secretion and exhaustion, in patients with chronic lymphocytic leukemia (CLL), offering an optimized strategy for better battling tumors. The IL-15 portion linked to scFvs specifically acts on NK cells, inducing NK cell activation and proliferation whilst avoiding off-target cytotoxicity [246]. TriKEs could also target C-type lectin domain family 12 member A (CLEC12A) in AML, showing superior efficacy compared to targeting CD33 [247]. Engineering T cells to express CARs targeting GPC2 and simultaneously secreting GD2 × CD16a bispecific innate immune cell engagers (BiCEs) could fuel the anti-cancer activity of immunotherapy [248]. GPC2 and GD2 are both targets of neuroblastoma, whereas CD16a is expressed on innate immune cells, namely NK cells or macrophages, within the TME. This approach, by simultaneously targeting two TAAs (GPC2/GD2) and secreting BiCEs to activate bystander innate immune cells, offers a potent and multifaceted strategy for treating neuroblastoma. In a nutshell, multispecific cell engagers offer a more comprehensive range of antigen-targeting possibilities

and more sophisticated signaling capabilities, which collectively enhance anti-tumor capabilities. Furthermore, development of this multispecific platform will address the limitations of conventional bispecific cell engager signaling and demonstrate a more efficacious avenue for activating and targeting immune effector cells.

The application of multi-antigen targeting cell strategies

OR-gate emphasizes broadening tumor recognition by selecting combinations of multiple TAAs with low co-expression levels in critical normal tissues. This approach aims to address the challenges of tumor heterogeneity and immune escape. Specifically, in hematological malignancies, well-validated targets such as CD19 and BCMA are frequently employed. Multi-targeting strategies often combine these established targets with other antigens to enhance tumor coverage and reduce the likelihood of recurrence. Examples include combinations such as CD19 ×CD123 [249], CD19 ×CD70 [250], and CD19 ×CD20 ×CD22 to prevent CD19-negative relapse [251]. The complex immunosuppressive TME in solid tumors presents more sophisticated challenges for target selection [252]. Combining TAAs that target different resistance mechanisms, such as pairing growth factor receptor targets with stromal targets or immune checkpoint targets, can overcome resistance mediated by the TME or immune suppression [253]. Combinations of TAAs targeting different resistance mechanisms, such as combining growth factor receptor targets with stromal or immune checkpoint targets, can overcome TME or immunosuppression-mediated resistance [253], e.g., PD-L1 ×CD19 [254], PD-L1 ×HER2 [255], PD-L1 ×GPC3 [256]. Introducing chemokine receptors that match the chemokines produced by the tumor conferred specific homing ability of CAR-T cells to the tumor, thereby increasing immune infiltration. Constructed EGFR/CXCR5 CAR-T cells demonstrated enhanced migration to CXCL13-positive tumors in preclinical models [257]. An early stage I clinical study is evaluating the therapeutic efficacy of EGFR/CXCR5 CAR-T cells (NCT05060796) in patients with NSCLC (often with high levels of secreted CXCL13). In AND-gate design, the combination of two TAAs with characteristic expression in tumors and limited expression in normal tissues could effectively improve the specificity and safety of treatment, such as CEA ×MSLN [258]. Some critical components of the TME in solid tumors often serve as limiting factors for targeting, such as CAFs. Combinations of the target FAP, which targets CAFs, with other TAAs have been explored, including FAP ×MSL [114, 259], FAP ×CLDN18 [260], and others. For NOT-gate design, iCAR targets should be selected based on their restricted expression in key normal tissues that need to be protected from off-target effects. For instance, in hematological malignancy treatment, CD93 is predominantly absent from non-hematopoietic tissues, and CD93-targeted CAR-T cells demonstrated efficacy in clearing AML in preclinical models [261]. However, human endothelial cells exhibit high expression of CD93, and the incorporation of iCAR to recognize endothelial cell-expressed CD19 significantly enhances the precision of CAR-T cell discrimination [261]. A promising and versatile strategy involves targeting HLA molecules via iCAR, which addresses the loss of heterozygosity phenomenon prevalent in tumor cells. This approach has been employed in several studies in combination with various TAAs [200, 202, 262, 263].

Multi-specific antibodies and cell engagers offer similar advantages as OR logic-gated CARs, reducing the potential for tumor escape by engaging multiple TAAs, but each has its own functional focus [264]. Multi-specific antibodies emphasize the incorporation of additional functional modules, and the selection of their targeted TAAs is designed to leverage the intrinsic functional properties of the antibody itself to enhance therapeutic efficacy. A notable example is the FDA-approved bispecific antibody, Amivantamab (EGFR/cMET BsAb), which is used for treating non-small cell lung cancer [265]. Amivantamab blocks EGFR-cMET receptor activation, facilitates receptor degradation, and triggers Fc effector-mediated ADCC [265]. Clinical trials in NSCLC are currently assessing the efficacy of a bispecific antibody designed for simultaneous dual immune checkpoint blockade (TIM-3/PD-1) [266] (NCT04931654). Additionally, the tri-specific antibody CS2009, targeting PD-1/VEGFA/CTLA-4, is undergoing Phase I clinical studies (NCT06741644). Cell engagers, conversely, are more inclined towards recruiting and activating endogenous immune cells to facilitate endogenous killing. Their TAA combinations are primarily focused on achieving efficient immune cell engagement and activation. Blinatumomab, the CD19 ×CD3 BiTE approved by the FDA for treating ALL, is a prime example. Clinical studies of TriKEs targeting HER2 ×CD16 ×NKG2D, which activates both NK cells and T cells, in patients with advanced solid tumors initially demonstrated good tolerability and promising anti-tumor activity [267].

Optimal TAA combinations for multi-targeting

Searching clinical trials on "ClinicalTrials.gov" reveals a growing trend of multi-targeting gene-edited cells in CAR therapy over the past 5 years. More than 50 studies related to "dual CAR", "multi-specific CAR", "BsAbs", and "cell engagers" have been conducted, encompassing both hematological and solid tumors. These studies

converge on optimizing TAA combinations, including CD19, BCMA, HER2, CLDN18.2, EpCAM, PSMA, and other relevant candidates, which are overexpressed in various cancers such as hepatocellular carcinoma, breast cancer, pancreatic cancer, and prostate cancer. Multi-targeting strategies are geared towards bypassing the essential roadblocks associated with single-target approaches, such as antigen loss, tumor heterogeneity, and resistance, thereby potentially enhancing the efficacy and durability of immunotherapy. While preliminary clinical outcomes provide a new dawn for immunotherapy, additional research is imperative to comprehensively investigate and adress safety and long-term efficacy concerns.

Dual targets

In designing dual-targeted gene-edited immune cells, CD19, CD20, and BCMA remain the primary targets to attack hematological malignancy. For solid tumors, HER2, PSMA, EGFR, VEGF, and EpCAM are the prominent focuses, often combined with inhibitory immune checkpoints in multi-target designs. Numerous studies have integrated inhibitory immune checkpoints into multi-target designs, emphasizing the importance of blocking inhibitory signaling pathways like PD-1/PD-L1 to fuel exhausted T cells and neutralize the immunosuppressive effects within the TME [268, 269]. The NK cells targeting PD-L1 and MICA/B exhibited a synergistic enhancement in cytotoxic activity within lung cancer models, leading to a substantial inhibition of tumor progression, potentially through the extensive pyroptosis mechanism induced by CAR-NK cells [270]. Developing armored CARs integrating cytokine receptors constitutes an alternative strategy to counteract T-cell exhaustion. The localized delivery of CD44/CD133 dual CAR-T cells, engineered with IL7Rα armoring to specifically target glioma stem cells, elicited sustained tumor regression in GBM and effectively mitigated T-cell exhaustion [271]. This therapeutic approach is currently under investigation in Phase I clinical trials (NCT05577091). The Clinical trials concerning dual-target combinations are summarized in Table 5. Notably, substantial concerns have been voiced about the potential risks linked to unpredictable cell proliferation and toxicity. Ouyang et al. engineered PD-1 knockdown CAR-T cells by introducing anti-PD-1 short hairpin RNA (shRNA) into T cells, potentially alleviating cell exhaustion while concurrently mitigating safety concerns [272].

Multi-targets

Preliminary clinical data of multi-targeting strategies demonstrated improved specificity and affinity of CAR-immune cell therapy while reducing toxicity to normal tissues, showing encouraging results. Applying multi-targeting strategies to CAR could provide cancer patients with more accurate, efficacious, robust, and secure therapeutic options. A recent study conducted by Bubb et al. demonstrated the therapeutic potential of targeting hematopoietic cytokine receptors in AML, revealing a marked delay in tumor progression and extended survival in NSG mice engrafted with mixed HCR^+ leukemia cells through the development of trivalent CAR-T cells targeting KIT, MPL, and FLT3 [273]. Furthermore, Bubb and colleagues engineered a BiTE incorporating SCF, TPO, and FLT3LG triple ligands along with an anti-CD3 scFv as an alternative therapeutic strategy, which conferred a dose-dependent tumor suppressor effect on T cells in in vitro experiments [273]. Several ongoing studies are enrolling volunteers to investigate multi-specific CAR-T cell therapies, including target combinations such as CD19 ×CD20 ×CD22, mesothelin ×GPC3 ×GUCY2C, and CLDN6 ×GPC3 ×mesothelin ×AXL (Table 6). Moreover, over 10 studies have explored multi-specific antibodies and cell engagers targeting T cells and NK cells (Table 6). For example, a clinical trial is underway to evaluate the anti-tumor efficacy and bio-safety of SENTI-202, a three-input OR-NOT logic-gated product, in treating AML, $CD33^+$ and $FLT3^+$ hematological malignancies (NCT06325748).

Developing multi-targeting immune cell therapies has secured significant milestones in hematological malignancy treatment, including ALL, AML, non-Hodgkin lymphoma (NHL) and diffuse large B-cell lymphoma (DLBCL), resulting in slowed tumor occurrence and prolonged patient survival. Clinical trials are currently being conducted for solid tumors, including pancreatic, prostate, breast, liver, ovarian, and cervical cancers (Table 7), with some preliminary positive outcomes. Besides, the multi-targeting strategy is not solely confined to treating neoplastic diseases but has also been extended to managing autoimmune disorders and viral infections, with related clinical trials currently underway or pending (NCT06548620, NCT06451159, NCT04648046). Crucially, multi-targeting strategies still present diverse challenges, including antigen selection accuracy, CAR design optimization, and strategy development to maintain efficacy while reducing treatment-related adverse effects.

CAR delivery methods: Viral and non-viral approaches

Transient modification

Transient expression of CAR

To completely eradicate all cancer cells, it is essential to ensure stable and persistent CAR expression in engineered immune cells that circulate into the body [274]. Stable CAR expression allows sufficient time for immune cells to specifically target and eliminate all tumor cells,

Table 5 Dual targets combination of CAR-immune cells

Strategy	Target	NCT Number	Study Status	Conditions	Interventions	Phases
A. Bispecific CAR	CD19 × BCMA	NCT04877080	WITHDRAWN	BCL	BIOLOGICAL: Fast dual CAR-T Injection	I
		NCT04412889	UNKNOWN	MM	BIOLOGICAL: BCMA/CD19 dual-target CAR-T cell immunotherapy	EARLY_I
		NCT05412329	UNKNOWN	MM	BIOLOGICAL: GC012 F injection	I
		NCT04236011	UNKNOWN	MM	BIOLOGICAL: GC012 F injection	EARLY_I
		NCT04617704	UNKNOWN	MM	BIOLOGICAL: GC012 F injection	EARLY_I
	CD19 × CD20	NCT04723914	UNKNOWN	R/R BCL	BIOLOGICAL: Dual target CAR-T cell therapy	I/II
		NCT04260932	COMPLETED	BCL	BIOLOGICAL: CD19/CD20 Dual-CAR-T cells	I
		NCT04260945	COMPLETED	BCL	BIOLOGICAL: CD19/CD20 Dual-CAR-T cells	I
		NCT04697290	SUSPENDED	B-NHL	BIOLOGICAL: CD19/CD20 Dual-CAR-T cells	EARLY_I
		NCT04486872	UNKNOWN	Diffuse Large B-Cell Lymphoma	BIOLOGICAL: Autologous humanized anti-CD19 and anti-CD20 dual specific CAR-T Cells	I
		NCT06395870	RECRUITING	R/R B-NHL	BIOLOGICAL: LUCAR-G39D cells product	I
		NCT06295549	RECRUITING	R/R B-NHL	BIOLOGICAL: LUCAR-G39P cells product	I
		NCT06503094	RECRUITING	BCL	DRUG: CD19&CD20 bispecific CAR-T cells	I/II
		NCT03271515	UNKNOWN	Leukemia, Lymphoma	BIOLOGICAL: Anti-CD19 anti-CD20 Bispecific CAR-T	I
		NCT04697940	RECRUITING	R/R B-NHL, Decitabine-primed Tandem CD19/CD20 CAR T Cells	BIOLOGICAL: Decitabine-primed Tandem CAR19/20 engineered T cells, DRUG: Fludarabine, DRUG: Cyclophosphamide	I/II
		NCT04553393	UNKNOWN	R/R Aggressive B-NHL With Huge Tumor Burden	DRUG: Chidamide DRUG: Decitabine DRUG: Chidamide and Decitabine BIOLOGICAL: Decitabine-primed Tandem CAR19/20 engineered T cells	I/II
	CD19 × CD22	NCT04723901	UNKNOWN	B-ALL R/R B-ALL	BIOLOGICAL: Dual target CAR-T cell therapy	I/II
		NCT04303247	UNKNOWN	R/R BCL	BIOLOGICAL: CD19 and CD22 targeted CAR-T cells	EARLY_I
		NCT05225831	UNKNOWN	$CD19^+$ and $CD22^+$ B-ALL	BIOLOGICAL: Autologous CD19/CD22 chimeric antigen receptor T-cells DRUG: Cyclophosphamide DRUG: Fludarabine	EARLY_I
		NCT06081478	RECRUITING	B-ALL, BCL, DLBCL	DRUG: CD19/CD22-bispecific CAR-T cells	II
	CD33 × CLL1	NCT05016063	UNKNOWN	AML	DRUG: Fludarabine DRUG: Cytoxan BIOLOGICAL: Dual CD33-CLL1 CAR-T cells	EARLY_I
		NCT05248685	UNKNOWN	AML	BIOLOGICAL: Dual CD33/CLL1 CAR T	I
	CLDN18.2 × PD-L1	NCT06084286	NOT_YET_RECRUITING	Advanced Solid Tumor	BIOLOGICAL: Dual-targeting CLDN18.2 and PD-L1 CAR-T cells	I
	CD38 × CLL1	NCT06110208	RECRUITING	AML	DRUG: CLL1 and CD38 dual-target CAR-T injection	EARLY_I

Table 5 (continued)

Strategy	Target	NCT Number	Study Status	Conditions	Interventions	Phases
	HER2 × PD-L1	NCT04684459	RECRUITING	Peritoneal Carcinoma Metastatic Pleural Effusion Malignant	BIOLOGICAL: Dual-targeting HER2 and PD-L1 CAR-T cells	EARLY_I
	NKG2D × NKp44	NCT05976906	RECRUITING	Advanced Solid Tumors	BIOLOGICAL: Universal chimeric natural killer receptor modified T-cells (CNK-UT)	I
	BCMA × CS1	NCT04662099	RECRUITING	$CS1^{+}$/$BCMA^{+}$ MM	BIOLOGICAL: Conditioning chemotherapy followed by CAR T cell infusion	I
	CD3 × CD20	NCT04703686	ACTIVE_NOT_RECRUITING	DLBCL, Refractory Indolent Adult NHL, Refractory Transformed B-NHL, Refractory Primary Mediastinal Large B-Cell Lymphoma, Refractory Mantle Cell Lymphoma	DRUG: Obinutuzumab DRUG: RO7082859	II
B. BsAb	PD-1 × CTLA-4	NCT04220307	COMPLETED	Nasopharyngeal Carcinoma	BIOLOGICAL: AK104	II
		NCT05559541	RECRUITING	Solid Tumor, Adult	DRUG: AK119, DRUG: AK104	I/II
		NCT04868708	COMPLETED	Recurrent or Metastatic Cervical Cancer	BIOLOGICAL: AK104 BIOLOGICAL: Bevacizumab DRUG: Paclitaxel DRUG: Cisplatin or Carboplatin	II
		NCT04380805	COMPLETED	Recurrent Cervical Cancer, Metastatic Cervical Cancer	BIOLOGICAL: AK104	II
		NCT06404736	NOT_YET_RECRUITING	Early Breast Cancer, Neoadjuvant Therapy, Triple Negative Breast Cancer	DRUG: Bispecific antibody (bsAb) targeting PD-1 and CTLA-4 DRUG: Albumin-bound paclitaxel DRUG: Carboplatin	II
		NCT05812534	NOT_YET_RECRUITING	Non-Small Cell Lung Cancer, Brain Metastases	DRUG: Cadonilimab	II
	PD-1 × VEGF	NCT04841538	WITHDRAWN	Thoracic Tumors, Non-small Cell Lung Cancer, SCLC	DRUG: ES101	I/II
		NCT04597541	ACTIVE_NOT_RECRUITING	Solid Tumor, Adult	DRUG: AK112	I/II
	PD-L1 × TGF-β	NCT05028556	ACTIVE_NOT_RECRUITING	Metastatic or Locally Advanced Solid Tumors	DRUG: Cohort 1 of Y101D DRUG: Cohort 2 of Y101D DRUG: Cohort 3 of Y101D DRUG: Cohort 4 of Y101D DRUG: Cohort 5 of Y101D	I
		NCT04954456	UNKNOWN	Advanced Malignant Tumor	DRUG: QLS31901	I
	EGFR × HER3	NCT04603287	RECRUITING	Locally Advanced or Metastatic Epithelial Tumor	DRUG: SI-B001	I
		NCT04900363	RECRUITING	NSCLC	DRUG: AK112	I/II
	CD3 × EpCAM	NCT06432296	RECRUITING	Malignant Ascites	DRUG: M701, PROCEDURE: paracentesis	III
		NCT06147037	RECRUITING	Advanced Solid Tumor, Metastatic Colorectal Carcinoma, Head and Neck Squamous Cell Carcinoma, NSCLC, Pancreatic Ductal Adenocarcinoma	DRUG: FPI-2053 DRUG: [111In]-FPI-2107 DRUG: [225 Ac]-FPI-2068	I
	CD3 × CD33	NCT05077423	TERMINATED	AML, Childhood	DRUG: CD33 × CD3 BsAb	I
	CD3 × PD-L1	NCT05938296	RECRUITING	Solid Tumor	DRUG: BS006 Injection	I
	PD-L1 × CTLA4	NCT04601610	TERMINATED	Advanced HCC	Drug: KN046 (PD-L1/CTLA4 BsAb) Drug: Ningetinib Tosylate(multi-target TKI)	I/II

Table 5 (continued)

Strategy	Target	NCT Number	Study Status	Conditions	Interventions	Phases
	PD-1 × LAG-3	NCT05577182	RECRUITING	Advanced Malignancies	DRUG: INCA32459-101	I
	PD-L1 × 4-1BB	NCT05159388	ACTIVE_NOT_RECRUITING	Solid Tumor	DRUG: PRS-344/S095012	I/II
	PD-L1 × LILRB2	NCT06259552	RECRUITING	Solid Tumor, HNSCC, RCC, CRC	BIOLOGICAL: SPX-303 Injection	I
	PD-L1 × VEGF	NCT05650385	NOT_YET_RECRUITING	Neoplasms Malignant	DRUG: B1962	I
	PD-L1 × CD27	NCT04440943	COMPLETED	NSCLC, Breast Cancer, Gastric Cancer, RCC, Ovarian Cancer, Primary Peritoneal Carcinoma, Fallopian Tube Cancer, Cholangiocarcinoma, Bladder Urothelial Carcinoma, MSI-H Colorectal Cancer, Esophageal Cancer, Hepatic Cancer, Head and Neck Cancer, Other Solid Tumors	DRUG: CDX-527	I
	PD-L1 × CD47	NCT05780307	RECRUITING	Advanced Solid Tumor, NSCLC, SCLC, Breast Cancer, HNSCC, CRC	DRUG: IMM2520	I
	CD3 × CD20	NCT06464185	RECRUITING	B-NHL	DRUG: Bispecific antibody-based combined with CAR-T cell therapy	I/II
	CD39 × TGF-β	NCT05381935	WITHDRAWN	Advanced Solid Tumor	DRUG: ES014, DRUG: ES014	I
	CD3 × CD276	NCT05999396	RECRUITING	CRC	DRUG: Administration of CC-3	I
	CD47 × DLL3	NCT05652686	RECRUITING	SCLC, Large Cell Neuroendocrine Cancer, Neuroendocrine Prostate Cancer, Gastroenteropancreatic Neuroendocrine Carcinoma (GEP-NEC)	DRUG: PT217	I/II
	CD3 × PSMA	NCT04496674	TERMINATED	Lung Cancer Squamous Cell	DRUG: CC-1 and Toczilizumab	I/II
	TIGIT × PDL1	NCT05102214	RECRUITING	Locally Advanced or Metastatic Solid Tumors, NSCLC	DRUG: HLX301	I/II
	CD47 × HER2	NCT05076591	RECRUITING	Advanced Solid Tumor, Advanced Breast Cancer, Advanced Gastric Cancer	DRUG: IMM2902	I
	CLDN18.2 × 4-1BB	NCT04900818	RECRUITING	Solid Tumor, Advanced Cancer, Metastatic Cancer, Gastric Cancer, Gastroesophageal Junction Carcinoma, Esophageal Adenocarcinoma	DRUG: TJ033721 DRUG: TJ033721, nivolumab, chemotherapy	I
C. Cell Engager	CD3 × CD19	NCT04556084	RECRUITING	B-ALL, R/R B-ALL	DRUG: Blinatumomab	II
		NCT06533579	NOT_YET_RECRUITING	B-ALL	GENETIC: Dose Level 1, VNX-101 GENETIC: Dose Level 2, VNX-101 GENETIC: Dose Level 3, VNX-10 , GENETIC: Dose Level 4, VNX-101	I/II
		NCT06220487	RECRUITING	Acute Lymphoblastic Leukemia, Philadelphia Chromosome, Philadelphia-Positive ALL, Adult ALL, IKZF1 Gene Mutation	DRUG: Prednisone, Olverembatinib, Blinatumomab, Chidamide	II
		NCT03628053	WITHDRAWN	ALL	BIOLOGICAL: Tisagenlecleucel DRUG: Blinatumomab DRUG: Inotuzumab	III
		NCT04827745	ACTIVE_NOT_RECRUITING	Mixed Phenotype Acute Leukemia, Measurable Residual Disease	DRUG: BLINCYTO (Blinatumomab)	II
		NCT04506086	RECRUITING	B-precursor ALL	DRUG: Blinatumomab DEVICE: Current Wearable Heatlth Monitoring System (CWHMS)	IV

Table 5 (continued)

Strategy	Target	NCT Number	Study Status	Conditions	Interventions	Phases
	CD19 × CD20	NCT06045910	RECRUITING	Lymphoma, NHL, DLBCL, Mantle-Cell, Lymphoma, Follicular	DRUG: ALETA-001	I/II
		NCT06202885	ACTIVE_NOT_RECRUITING	Lymphoma	DRUG: Immunotherapy	
	CD3 × EFGR	NCT04903795	NOT_YET_RECRUITING	Malignant Glioma, Glioblastoma	DRUG: hEGFRvIII-CD3 (BRiTE)	I
		NCT05387265	RECRUITING	Solid Tumors	DRUG: CX-904	I
		NCT06502977	NOT_YET_RECRUITING	Advanced SCLC	DRUG: Tarlatamab	II
	CD3 × DLL3	NCT05882058	RECRUITING	SCLC, Neuroendocrine Neoplasms	DRUG: BI 764532, formulation 1 DRUG: BI 764532, formulation 2	II
		NCT04702737	ACTIVE_NOT_RECRUITING	Neuroendocrine Prostate Cancer	DRUG: Tarlatamab	I
		NCT05361395	RECRUITING	Extensive Stage SCLC	DRUG: Tarlatamab DRUG: Carboplatin DRUG: Etoposide DRUG: Atezolizumab DRUG: Durvalumab	I
	CD3 × CLDN6	NCT06276491	RECRUITING	Ovarian Cancer, Endometrial Cancer, Germ Cell Tumor, Testicular Germ Cell Tumor, Ovarian Germ Cell Tumor	BIOLOGICAL: XmAb541	I
	CD8/TCR × CD20	NCT06542250	NOT_YET_RECRUITING	B-cell Malignancies	DRUG: AZD5492	I/II
	CD3 × PSMA	NCT04631601	TERMINATED	Metastatic Castration-resistant Prostate Cancer	DRUG: Acapatamab DRUG: Enzalutamide DRUG: Abiraterone DRUG: AMG 404	I/II
	CD3 × CLDN18.2	NCT04260191	TERMINATED	Gastric and Gastroesophageal Junction Adenocarcinoma	DRUG: AMG 910	I
	CD3 × FLT3	NCT05143996	RECRUITING	R/R AML, MDS	DRUG: CLN-049	I
	CD3 × CD38	NCT05908396	TERMINATED	MM	DRUG: IGM-2644	I
	CD3 × MCU17	NCT04117958	TERMINATED	MUC17-positive Solid Tumors	DRUG: AMG 199	I
	CD3 × TYRP1	NCT04551352	COMPLETED	Cutaneous Melanoma Uveal Melanoma Mucosal Melanoma	DRUG: RO7293583 DRUG: Tocilizumab DRUG: Obinutuzumab DRUG: Adalimumab	I
	CD3 × gp100	NCT06070012	NOT_YET_RECRUITING	Uveal Melanoma	DRUG: Tebentafusp	II
	CD3 × EpCAM	NCT05808634	RECRUITING	Advanced Adenocarcinoma	DRUG: BA3182	I
	CD3 × STEAP1	NCT04221542	RECRUITING	Prostate Cancer	DRUG: AMG 509 DRUG: Abiraterone DRUG: Enzalutamide	I

Table 5 (continued)

Strategy	Target	NCT Number	Study Status	Conditions	Interventions	Phases
	CD30 × CD16 A	NCT04074746	ACTIVE_NOT_RECRUITING	Recurrent Anaplastic Large Cell Lymphoma, R/R B-NHL, Recurrent Classic Hodgkin Lymphoma, Recurrent Mycosis Fungoides, Recurrent Peripheral T-Cell Lymphoma, Not Otherwise Specified, Refractory Anaplastic Large Cell Lymphoma, Refractory Classic Hodgkin Lymphoma, Refractory Mycosis Fungoides, Refractory Peripheral T-Cell Lymphoma, Not Otherwise Specified	BIOLOGICAL: Anti-CD30/CD16 A Monoclonal Antibody AFM13 DRUG: Cyclophosphamide DRUG: Fludarabine DRUG: Fludarabine Phosphate BIOLOGICAL: Genetically engineered lymphocyte therapy	I/II
	CD16 × EGFR	NCT05099549	TERMINATED	Squamous Cell Carcinoma of Head and Neck Carcinoma, NSCLC; Colorectal Neoplasms; Advanced Solid Tumor, Refractory Tumor; Metastatic Tumor	DRUG: AFM24 BIOLOGICAL: SNK01	I/II
	CD16 × CD30	NCT05883449	RECRUITING	R/R Hodgkin Lymphoma, Peripheral T Cell Lymphoma	DRUG: AFM13 DRUG: AB-101 DRUG: Cyclophosphamide DRUG: Fludarabine DRUG: Interleukin-2	II
	Vδ2 × PSMA	NCT05369000	RECRUITING	Metastatic Castration Resistant Prostate Cancer	BIOLOGICAL: LAVA-1207 BIOLOGICAL: LAVA-1207 plus Pembrolizumab	I/II

BCL B-cell Lymphoma; *MM* multiple myeloma; *R/R* Relapsed/Refractory; *B-NHL* B-cell non-hodgkin's lymphoma; *B-ALL* B-cell acute lymphoblastic leukemia; *DLBCL* diffuse large B cell lymphoma; *AML* acute myeloid leukemia; *SCLC* small cell lung cancer; *NSCLC* Non-small cell lung cancer; *HNSCC* head and neck squamous cell carcinoma; *RCC* renal cell carcinoma; *CRC* colorectal cancer; *MDS* myelodysplastic syndrome

Table 6 Multi-target combination of CAR-immune cells

	Target	NCT Number	Study Status	Conditions	Interventions	Phases
A. Multi-specific CAR	Mesothelin × GPC3 × GUCY2 C	NCT05779917	RECRUITING	Pancreas Cancer, CAR-T Cell Therapy, Mesothelin, Solid Tumor, Adult	BIOLOGICAL: CAR-T cells	I
	PD1 × CTLA-4 × MSLN	NCT06248697	RECRUITING	Solid Tumor	BIOLOGICAL: αPD1/CTLA4-MSLN-CAR T cells	EARLY_I
	CLDN6 × GPC3 × Mesothelin × AXL	NCT05410717	RECRUITING	Stage IV Ovarian Cancer, Testis Cancer, Refractory, Endometrial Cancer Recurrent, CAR NK	BIOLOGICAL: Claudin6, GPC3, Mesothelin, or AXL targeting CAR-NK cells	I
	CD19 × CD20 × CD22	NCT05418088	RECRUITING	Recurrent ALL, Recurrent B-ALL, Recurrent B-Cell Prolymphocytic Leukemia, 13 more	BIOLOGICAL: Anti-CD19/CD20/CD22 CAR T-Cells DRUG: Cyclophosphamide DRUG: Fludarabine Phosphate	I
	CD19 × EGFR × IL-12	NCT06343376	RECRUITING	Recurrent Chronic Lymphocytic Leukemia, Recurrent DLBCL, Recurrent Follicular Lymphoma, 11 more	BIOLOGICAL: EGFRt/19-28z/IL-12 CAR T-lymphocytes, DRUG: Fludarabine Phosphate, PROCEDURE: Leukapheresis PROCEDURE: Multigated Acquisition Scan PROCEDURE: Positron Emission Tomography 7 more	I
	CD19 × CD20 × CD22	NCT05010564	RECRUITING	Leukemia, B-Cell	GENETIC: Autologous TRICAR-ALL T-cells and lymphodepletion chemotherapy	I
	B7-H3 × EGFR × HER2 × IL13-zetakine	NCT05768880	RECRUITING	Diffuse Intrinsic Pontine Glioma, Diffuse Midline Glioma, Recurrent CNS Tumor, Adult, Recurrent, CNS Tumor, Childhood, Refractory Primary Malignant Central Nervous System Neoplasm	BIOLOGICAL: SC-CAR4BRAIN	I
B. Multi-specific antibody	4-1BB × PD-L1 × HSA	NCT04442126	TERMINATED	Advanced Solid Tumor, NSCLC, CRC, Squamous Cell Carcinoma, Ovarian Carcinoma, Peritoneal Carcinoma, Fallopian Tube Cancer, HNSCC, Triple Negative Breast Cancer	BIOLOGICAL: NM21-1480	I/II
	CD3 × 4-1BB × PD-L1 × ROR1	NCT05104775	RECRUITING	Hematologic Malignancies	DRUG: GNC-035	I
		NCT05039931	WITHDRAWN	Breast Cancer, Solid Tumor	DRUG: GNC-035	I
		NCT05160545	RECRUITING	Breast Cancer	DRUG: GNC-035	I
	CD3 × CD19 × 4-1BB × PD-L1	NCT05192486	RECRUITING	DLBCL	DRUG: GNC-038	I/II
	CD3 × 4-1BB × PD-L1 × EGFRVIII	NCT04794972	RECRUITING	Recurrent Glioma, Solid Tumor	DRUG: GNC-039	I
C. Multi-specific Cell Engager	CD3 × CD19 × CD20	NCT05987605	NOT_YET_RECRUITING	Advanced Malignancies	DRUG: 1 A46 Drug Substance	I
	CD123 × NKp46 × CD16a	NCT06508489	RECRUITING	AML	BIOLOGICAL: SAR443579, DRUG: venetoclax, DRUG: azacitidine	I/II
	NKp46 × CD16 × CD20 × IL-2 receptor	NCT06088654	RECRUITING	NHL	DRUG: IPH6501	I/II

Table 6 (continued)

	Target	NCT Number	Study Status	Conditions	Interventions	Phases
	CD3 × DLL3 × albumin	NCT04471727	RECRUITING	Small-Cell Lung Cancer, Neuroendocrine Carcinoma	BIOLOGICAL: MK-6070 BIOLOGICAL: Atezolizumab BIOLOGICAL: Ifinatamab Deruxtecan (I-DXd)	I/II
	CD3 × CD19 × CD20	NCT05348889	RECRUITING	NHL, ALL, Disease (Disorder)	DRUG: 1 A46 Injection	I/II
	CD16 × CD33 × IL-15	NCT03214666	TERMINATED	High-risk Myelodysplastic Syndromes, AML, Systemic Mastocytosis, Mast Cell Leukemia	DRUG: GTB-3550 TriKE Phase I, DRUG: GTB-3550 TriKE Phase II	I/II
	BCMA × CD16 × NKp46	NCT05839626	RECRUITING	R/R MM, Amyloid Light-chain Amyloidosis	DRUG: SAR445514	I/II
	CD19 × CD3 × HSA	NCT05879744	ACTIVE_NOT_RECRUITING	NHL, Relapsed, Adult	DRUG: CLN-978	I
	CD3 × CD33 × CD123 × CD70	NCT05673057	RECRUITING	Leukemia, Relapsed, Myeloid, Acute	DRUG: MP0533 (multi-specific DARPIN CD3 Engager Targeting CD33, CD123 and CD70)	I/II

ALL acute lymphoblastic leukemia; *B-ALL* B-cell acute lymphoblastic leukemia; *DLBCL* diffuse large B cell lymphoma; *CNS* central nervous system; *NSCLC* Non-small cell lung cancer; *CRC* colorectal cancer; *HNSCC* head and neck squamous cell carcinoma; *AML* acute myeloid leukemia; *NHL* non-hodgkin lymphoma; *MM* multiple myeloma

Table 7 Current clinical applications in disease

Disease	NCT Number	Study Title	Study Status	Phases
A. Tumor				
HER2-positive Gastric Cancer HER2-positive Breast Cancer	NCT04650451	Safety and Activity Study of HER2-Targeted Dual Switch CAR-T Cells (BPX-603) in Subjects with HER2-Positive Solid Tumors	SUSPENDED	I
B-cell Lymphoma	NCT04260932	CD19/CD20 Dual-CAR-T in B-cell Lymphoma Patients	COMPLETED	I
B-cell Leukemia	NCT04260945	CD19/CD20 Dual-CAR-T in B-cell Leukemia Patients	COMPLETED	I
Peritoneal Carcinoma	NCT04684459	Dual-targeting HER2 and PD-L1 CAR-T for Cancers with Pleural or Peritoneal Metastasis	RECRUITING	EARLY_I
B-cell Lymphoma	NCT04303247	CD19 and CD22 Dual-targeted CAR-T Cells for Relapsed or Refractory B-NHL	UNKNOWN	EARLY_I
B-cell Non-Hodgkin's Lymphoma	NCT04697290	CD19/CD20 Dual-CAR-T in B-cell Non-Hodgkin's Lymphoma Patients	SUSPENDED	EARLY_I
Recurrent Glioblastoma	NCT05577091	Tris-CAR-T Cell Therapy for Recurrent Glioblastoma	RECRUITING	I
Ovarian Cancer Cervix Cancer Gynecologic Cancer	NCT06215950	A Clinical Research About CD70-targeted CAR-T in the Treatment of CD70-positive Advanced/Metastatic Gynecologic Cancer	RECRUITING	I
Renal Cell Carcinoma Ovarian Cancer Cervix Cancer	NCT06010875	A Clinical Research About CD70-targeted CAR-T in the Treatment of CD70-positive Advanced/Metastatic Solid Tumors	RECRUITING	I
Glioblastoma Multiforme of Brain	NCT05627323	CAR T Cells in Patients with $MMP2^+$ Recurrent or Progressive Glioblastoma	ACTIVE_NOT_RECRUITING	I
Pediatric Solid Tumor Germ Cell Tumor Retinoblastoma Hepatoblastoma Wilms Tumor Rhabdoid Tumor Carcinoma Osteosarcoma Ewing Sarcoma Rhabdomyosarcoma Synovial Sarcoma Clear Cell Sarcoma Malignant Peripheral Nerve Sheath Tumors Desmoplastic Small Round Cell Tumor Soft Tissue Sarcoma Neuroblastoma	NCT03618381	EGFR806 CAR T Cell Immunotherapy for Recurrent/Refractory Solid Tumors in Children and Young Adults	RECRUITING	I
Acute Myeloid Leukemia	NCT06110208	Study to Evaluate the Safety and Preliminary Efficacy of CLL1 and CD38 Dual CAR-T in R/R AML	RECRUITING	EARLY_I
Large B-cell Lymphoma Diffuse Large B-Cell Lymphoma	NCT04889716	CAR-T Followed by Bispecific Antibodies	RECRUITING	II
Advanced Sarcoma	NCT06087341	A Phase I Trial of Memory T Cells Expressing an NKG2D Chimeric Antigen Receptor in Children, Adolescents, and Young Adults With Advanced Sarcoma	RECRUITING	I
Recurrent Glioblastoma Recurrent Malignant Glioma Recurrent WHO Grade II Glioma Recurrent WHO Grade III Glioma	NCT04214392	Chimeric Antigen Receptor (CAR) T Cells With a Chlorotoxin Tumor-Targeting Domain for the Treatment of MMP2 + Recurrent or Progressive Glioblastoma	RECRUITING	I
Multiple Myeloma in Relapse Multiple Myeloma, Refractory	NCT04861480	Dual-target CAR-T Cells (C-4–29) in the Treatment of Relapsed/Refractory Multiple Myeloma	ENROLLING_BY_INVITATION	I
Diffuse Large B Cell Lymphoma	NCT04486872	An Exploratory Clinical Trial of Autologous Humanized Anti-cluster of Differentiation Antigen 19/20(CD19/CD20) Dual Specific CAR-T Cells Injection	UNKNOWN	I

Table 7 (continued)

Disease	NCT Number	Study Title	Study Status	Phases
Prostate Cancer	NCT03089203	CART-PSMA-TGFβRDN Cells for Castrate-Resistant Prostate Cancer	ACTIVE_NOT_RECRUITING	I
High-grade Glioma WHO Grade IV Glioma	NCT06482905	Safety and Efficacy Study of TX103 CAR-T Cell Therapy for Recurrent or Progressive Grade 4 Glioma	NOT_YET_RECRUITING	I
Non-hodgkin Lymphoma	NCT06323525	TCR Reserved and Power3 Gene Knock-out Allogeneic CD19-targeting CAR-T Cell Therapy in r/r B Cell Lymphoma	RECRUITING	I|II
Chronic Myelomonocytic Leukemia	NCT06071624	Chimeric Antigen Receptor T Cell Therapy Redirected to CD4 (CD4 CAR) as a Second Line Treatment for Chronic Myelomonocytic Leukemia, CMML	RECRUITING	I
Pancreatic Cancer	NCT04183478	The Efficacy and Safety of K-001 in the Treatment of Advanced Pancreatic Cancer	UNKNOWN	II|III
Advanced Non-Small Cell Lung Cancer	NCT05785767	A Study to Learn if a Combination of Fianlimab and Cemiplimab Versus Cemiplimab Alone is More Effective for Adult Participants with Advanced Non-Small Cell Lung Cancer (NSCLC)	RECRUITING	II|III
Non-Small Cell Lung Cancer	NCT05800015	A Trial to Learn How the Combination of Fianlimab with Cemiplimab and Chemotherapy Works Compared with Cemiplimab and Chemotherapy for Treating Adult Patients with Advanced Non-small Cell Lung Cancer	RECRUITING	II|III
Neuroblastoma Osteosarcoma Other Solid Tumor Cancers	NCT03860207	Study of the Safety and Efficacy of Humanized 3 F8 Bispecific Antibody (Hu3 F8-BsAb) in Patients with Relapsed/Refractory Neuroblastoma, Osteosarcoma, and Other Solid Tumor Cancers	TERMINATED	I|II
Locally Advanced and Metastatic Pancreatic Cancer	NCT04324307	Study of Immunotherapy Combined With Chemotherapy in Locally Advanced and Metastatic Pancreatic Cancer	RECRUITING	I|II
Acute Myeloblastic Leukaemia	NCT05077423	A Phase 1 Trial of CD33xCD3 BsAb in Pediatric Patients with Relapsed or Refractory Acute Myeloid Leukemia	TERMINATED	I
Advanced or Metastatic Esophageal Squamous Cell Carcinoma	NCT04785820	A Study of Lomvastomig (RO7121661) and Tobemstomig (RO7247669) Compared with Nivolumab in Participants with Advanced or Metastatic Squamous Cell Carcinoma of the Esophagus	ACTIVE_NOT_RECRUITING	II
Leukemia Lymphoma	NCT00014560	Antibody Therapy in Treating Patients with Refractory or Relapsed Non-Hodgkin's Lymphoma or Chronic Lymphocytic Leukemia	TERMINATED	I
Follicular Lymphoma	NCT05529524	Signatures of Response and Resistance to Mosunetuzomab in Follicular Lymphomas	RECRUITING	
Small Cell Lung Cancer Large Cell Neuroendocrine Cancer Neuroendocrine Prostate Cancer Gastroenteropancreatic Neuroendocrine Carcinoma	NCT05652686	A Study of PT217 in Patients with Neuroendocrine Carcinomas Expressing DLL3 (the SKYBRIDGE Study)	RECRUITING	I|II
Lung Cancer Squamous Cell	NCT04496674	Bispecific PSMAxCD3 Antibody CC-1 in Patients with Squamous Cell Carcinoma of the Lung	TERMINATED	I|II
Castration-Resistant Prostatic Cancer	NCT04104607	the Bispecific PSMAxCD3 Antibody CC-1 in Patients with Castration Resistant Prostate Carcinoma	RECRUITING	I
B-cell Non-Hodgkin Lymphoma	NCT05991388	A Global Study of Novel Agents in Paediatric and Adolescent Relapsed and Refractory B-cell Non-Hodgkin Lymphoma	ACTIVE_NOT_RECRUITING	II|III

Table 7 (continued)

Disease	NCT Number	Study Title	Study Status	Phases
Glioblastoma Astrocytoma, Grade IV	NCT02766699	A Study to Evaluate the Safety, Tolerability and Immunogenicity of EGFR(V)-EDV-Dox in Subjects with Recurrent Glioblastoma Multiforme (GBM)	UNKNOWN	I
Advanced Solid Tumor Metastatic Colorectal Carcinoma Head and Neck Squamous Cell Carcinoma Non-small Cell Lung Cancer Pancreatic Ductal Adenocarcinoma	NCT06147037	A Phase 1, Dose-escalation Study of [225 Ac]-FPI-2068 in Adult Patients with Advanced Solid Tumours	RECRUITING	I
Non-Small Cell Lung Cancer Brain Metastases	NCT05812534	Study of Cadonilimab Combined with Bevacizumab and Chemotherapy for Advanced Nonsquamous Non-small Cell Lung Cancer Patients with Untreated Brain Metastases	NOT_YET_RECRUITING	II
Early Breast Cancer Neoadjuvant Therapy Breast Cancer	NCT06404463	QL1706 Plus Chemotherapy as Neoadjuvant Therapy in Early High-Risk $ER^{+}/HER2^{-}$ Breast Cancer	NOT_YET_RECRUITING	II
High-risk Myelodysplastic Syndromes Acute Myelogenous Leukemia Systemic Mastocytosis Mast Cell Leukemia	NCT03214666	GTB-3550 Tri-Specific Killer Engager (TriKE) for High-Risk Hematological Malignancies	TERMINATED	I|II
Malignant Glioma Glioblastoma	NCT04903795	Bispecific T Cell Engager BRiTE for Patients with Grade IV Malignant Glioma	NOT_YET_RECRUITING	I
Acute Myeloid Leukemia	NCT06508489	A Study to Investigate Natural Killer Cell Engager (SAR443579) With Different Agents in Participants with Hematological Malignancies	RECRUITING	I|II
Non-Hodgkin Lymphoma	NCT06088654	I/2 Study of IPH6501 in Patients with Relapsed/Refractory B-Cell Non-Hodgkin Lymphoma	RECRUITING	I|II
Diffuse Large B Cell Lymphoma	NCT05189782	Mechanism of Resistance to GNC-038 in Relapsed and Refractory Diffuse Large B-cell Lymphoma	RECRUITING	
Diffuse Large B-cell Lymphoma	NCT01741792	Clinical Study with Blinatumomab in Patients with Relapsed/Refractory Diffuse Large B-cell Lymphoma (DLBCL)	COMPLETED	II
Ovarian Cancer Endometrial Cancer Germ Cell Tumor Testicular Germ Cell Tumor Ovarian Germ Cell Tumor	NCT06276491	Phase 1, Safety and Tolerability Study of XmAb541 in Advanced Solid Tumors	RECRUITING	I
Metastatic Castration-resistant Prostate Cancer	NCT03792841	Safety, Tolerability, Pharmacokinetics, and Efficacy of Acapatamab in Subjects With mCRPC	COMPLETED	I
Acute Lymphoblastic Leukemia Philadelphia Chromosome Philadelphia-Positive ALL Adult ALL IKZF1 Gene Mutation	NCT06220487	A Single-arm, Open-label Study of Olverembatinib, CD3/CD19 Bispecific T-cell Engager, and Chidamide in Patients with Newly Diagnosed Ph + ALL	RECRUITING	II
Small Cell Lung Carcinoma Neuroendocrine Neoplasms	NCT05882058	DAREON: A Study to Test Whether Different Doses of BI 764532 Help People with Small Cell Lung Cancer or Other Neuroendocrine Cancers	RECRUITING	II
B-cell Acute Lymphoblastic Leukemia	NCT01207388	Confirmatory Phase II Study of Blinatumomab (MT103) in Patients with Minimal Residual Disease of B-precursor Acute Lymphoblastic Leukemia (ALL)	COMPLETED	II
Lymphoma	NCT04074746	Modified Immune Cells (AFM13-NK) and A Monoclonal Antibody (AFM13) in Treating Patients with Recurrent or Refractory CD30-Positive Hodgkin or Non-Hodgkin Lymphomas	ACTIVE_NOT_RECRUITING	I|II

Table 7 (continued)

Disease	NCT Number	Study Title	Study Status	Phases
Non-Hodgkin's Lymphoma, Relapsed	NCT00274742	Safety Study of the Bispecific T-cell Engager Blinatumomab (MT103) in Patients with Relapsed NHL	COMPLETED	I
Squamous Cell Carcinoma of the Head and Neck Carcinoma Non-Small-Cell Lung Colorectal Neoplasms	NCT05099549	Safety, Tolerability, and Anti-Tumor Activity of AFM24 in Combination with SNK01 in Subjects with Advanced/Metastatic EGFR-Expressing Cancers	TERMINATED	I|II
Lymphoma	NCT06045910	A Phase I/II Trial of ALETA-001 for the Treatment of Participants With B-cell Malignancies	RECRUITING	I|II
Non-Hodgkin's Lymphoma Acute Lymphoid Leukemia, Disease	NCT05348889	First-in-Human (FIH) Trial of 1 A46 in Subjects with Advanced CD20 and/or CD19 Positive B-cell Hematologic Malignancies	RECRUITING	I|II
Pancreas Cancer Mesothelin	NCT05779917	Mesothelin/GPC3/GUCY2 C-CAR-T Cells Against Cancers	RECRUITING	I
Stage IV Ovarian Cancer Testis Cancer Endometrial Cancer	NCT05410717	CLDN6/GPC3/Mesothelin/AXL-CAR-NK Cell Therapy for Advanced Solid Tumors	RECRUITING	I
Acute Lymphoblastic Leukemia Recurrent B-Cell Prolymphocytic Leukemia	NCT05418088	Genetically Engineered Cells (Anti-CD19/CD20/CD22 CAR T-cells) for the Treatment of Relapsed or Refractory Lymphoid Malignancies	RECRUITING	I
B-Cell Leukemia	NCT05010564		RECRUITING	I
Advanced Solid Tumor Non-small Cell Lung Cancer Colorectal Cancer Squamous Cell Carcinoma Ovarian Carcinoma Peritoneal Carcinoma Fallopian Tube Cancer Head and Neck Squamous Cell Carcinoma Triple Negative Breast Cancer	NCT04442126	Trivalent CAR-T Cell in Acute B-Lineage Leukemia (TRICAR-ALL)	TERMINATED	I/II
Breast Cancer	NCT05039931	A Study of GNC-035, a Tetra-specific Antibody, in Participants with Locally Advanced or Metastatic Solid Tumors	WITHDRAWN	I
Breast Cancer	NCT05160545	A Study of GNC-035, a Tetra-specific Antibody, in Participants with Locally Advanced or Metastatic Breast Cancer	RECRUITING	I
Diffuse Large B-cell Lymphoma	NCT05192486	A Study of GNC-038, a Tetra-specific Antibody, in Participants With R/R Diffuse Large B-cell Lymphoma (DLBCL)	RECRUITING	I/II
Small-Cell Lung Cancer Neuroendocrine Carcinoma	NCT04471727	A Study in Participants with Advanced Cancers Associated with Expression of DLL3 (MK-6070–001/HPN328-4001)	RECRUITING	I/II
Multiple Myeloma	NCT05839626	A Study to Investigate Safety and Efficacy with SAR445514 in Participants with Relapsed/Refractory Multiple Myeloma (RRMM) and Relapsed/Refractory Light-chain Amyloidosis (RRLCA)	RECRUITING	I/II
B. Other Disease				
Systemic Lupus Sclerosis ANCA Associated Vasculitis Idiopathic Inflammatory Myopathies Multiple Sclerosis NMO Spectrum Disorder Myasthenia Gravis	NCT06548620	A Study of RD06-04 in Patients with Active Autoimmune Diseases	NOT_YET_RECRUITING	EARLY_I
Progressive Multiple Sclerosis	NCT06451159	A Study of KYV-101, a CD19 CAR T Cell Therapy, in Participants with Treatment Refractory Progressive Multiple Sclerosis	RECRUITING	I

Table 7 (continued)

Disease	NCT Number	Study Title	Study Status	Phases
Neuromyelitis Optica Spectrum Disorders Myasthenia Gravis, Generalized Multiple Sclerosis Chronic Inflammatory Demyelinating Polyradiculoneuropathy	NCT06485232	Universal CAR-T Cells in Patients with Refractory Autoimmune Diseases of the Nervous System	NOT_YET_RECRUITING	EARLY_I
HIV Infections	NCT04648046	CAR-T Cells for HIV Infection	RECRUITING	I|II
Autoimmune Diseases of the Nervous System Neuromyelitis Optica Spectrum Disorder Myasthenia Gravis, Chronic Inflammatory Demyelinating Polyradiculoneuropathy Idiopathic Inflammatory Myopathies Multiple Sclerosis Autoimmune Encephalitis Myelin Oligodendrocyte Glycoprotein Antibody-Associated Disease POEMS Syndrome	NCT04561557	Safety and Efficacy of CT103 A Cells for Relapsed/Refractory Antibody-associated Inflammatory Diseases of the Nervous System	RECRUITING	EARLY_I
Multiple Sclerosis, Primary Progressive Multiple Sclerosis, Secondary Progressive	NCT06138132	A Study of Anti-CD19 Chimeric Antigen Receptor T-Cell (CAR-T) Therapy in Subjects With Non-relapsing and Progressive Forms of Multiple Sclerosis	RECRUITING	I
Systemic Lupus Erythematosus	NCT03030976	A Study of CD19-Redirected Autologous T Cells for CD19-Positive Systemic Lupus Erythematosus (SLE)	UNKNOWN	I

while durable CAR expression provides a reliable guarantee against tumor recurrence. Conversely, in diseases beyond cancer, such as fibrosis, autoimmune disorders, metabolic diseases, and age-related diseases, CAR-T cell therapy could achieve a desired therapeutic effect by reducing the number of pathologically defective cells below the pathological threshold, rather than requiring complete eradication [118]. Transient CAR expression confers a more favorable therapeutic profile in the context of extratumoral diseases, especially enabling improved safety. While the conventional approach to generating CAR-T cells typically involves the permanent integration of a transgene through viral vector transduction, advances in RNA delivery technology now permit the transient expression of specific CARs by transfecting T cells with mRNA [275]. As mRNA doesn't integrate into the host genome, this approach avoids oncogenicity risk associated with random insertion of viral vectors [275, 276]. Moreover, transient CAR expression enjoys the characteristic of specific T cell targeting for a limited duration, thereby mitigating adverse reaction risks concerning sustained CAR expression [276]. Transient CAR expression allows for more precise and flexible control during the therapeutic process, enabling medical personnel to adjust therapeutic dosages on a case-by-case basis to manage potential toxicities. Advantageously, transient CAR expression reduces non-tumor-targeted toxicity and enhances biosafety [277]. Even if immune cells are redirected to healthy tissue via CAR-mediated targeting, the risk of off-target damage can be mitigated by the short duration of CAR expression [278]. In addition, programming T cells in vivo to generate CAR-T cells does not lead to cytokine release beyond physiological levels, as observed in ex vivo cultures, which could be attributed to the unaltered physiological environment of T cells. Furthermore, tumor-antagonizing immunity could be exerted without necessitating multiple rounds of in vitro expansion, a characteristic absent in isolated cultured CAR-T cells, which simplifies technical requirements and reduces production costs [277]. Nevertheless, transient CAR expression may attenuate the anti-cancer responses of T cells, thus necessitating repeated administrations to sustain stable therapeutic outcomes [278]. In a nutshell, transient CAR expression offers a safer and more flexible approach to CAR-T cell therapy, presenting clear advantages in risk reduction and enhanced treatment control, despite the repeated administration challenge.

Multiple strategies could achieve transient CAR expression in vivo, including delivery of mRNA encoding CAR, introduction of non-integrating vectors, and inducible CAR systems. By delivering lipid nanoparticles (LNPs) containing mRNA encoding CAR into cells, the translation of mRNA prior to its degradation enables CAR expression [42, 279]. Owing to the lack of CAR genetic information integration into the genome, effector cells lose CAR expression as a consequence of mRNA degradation (Fig. 7A). Selecting a delivery vector is crucial for determining the extent of transient

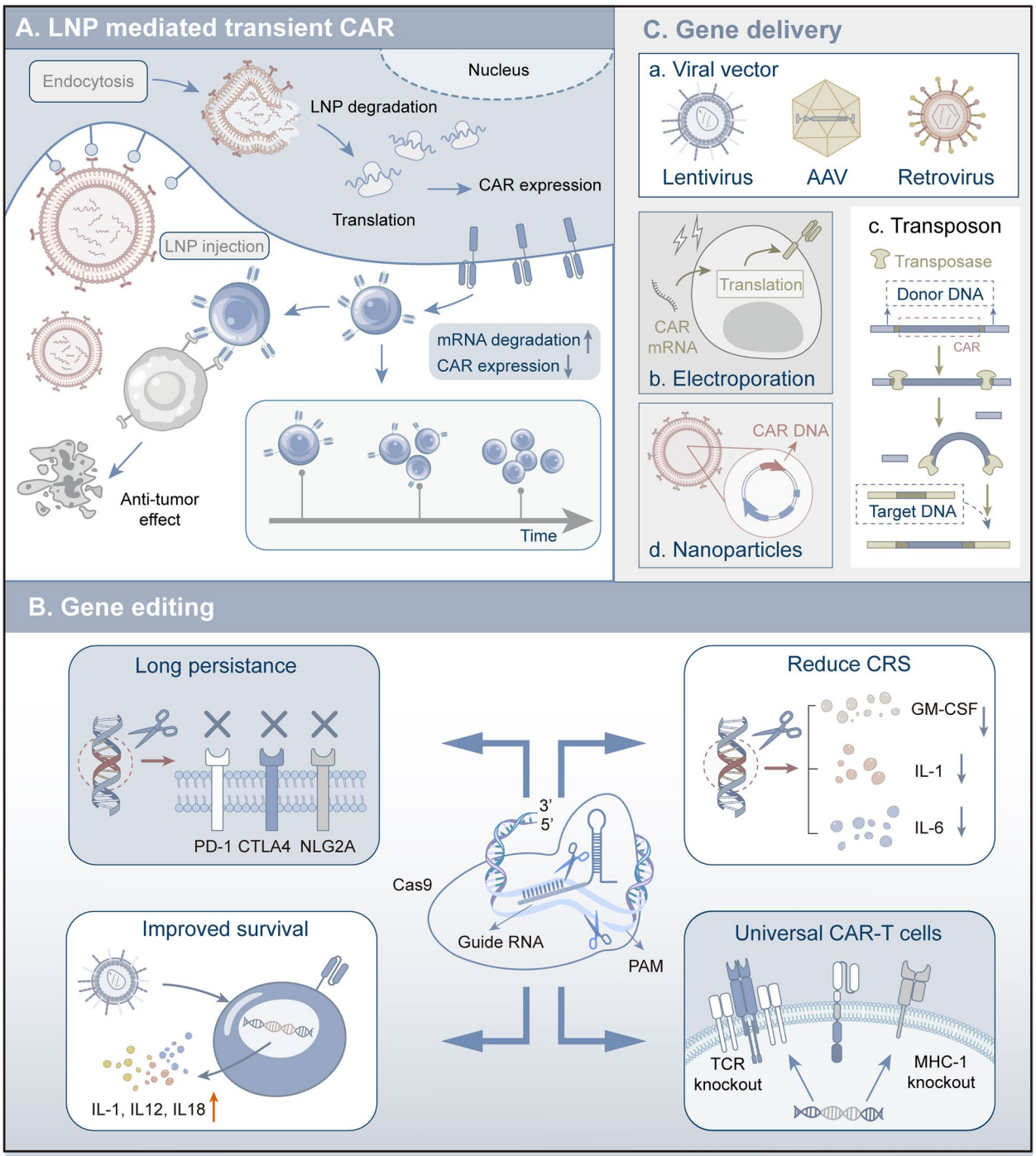

Fig. 7 Emerging novel strategies. **A**. Lipid nanoparticle (LNP)-mediated expression of CAR. LNPs enter cells via endocytosis and then degrade, releasing mRNA encoding the CAR, which is subsequently translated to promote CAR expression. Immune cells expressing CARs exert CAR-mediated anti-tumor effects, but this function gradually diminishes as the mRNA degrades. **B**. Application of gene editing technology in improving the function of immune cells **C**. Strategies for gene delivery a. Viral vectors. Lentivirus: Single-stranded RNA genome, enveloped, conical core capsid, capable of host genome integration. Adenovirus: Double-stranded linear DNA genome, non-enveloped, icosahedral capsid, episomal persistence without integration. Retrovirus: Single-stranded RNA genome, enveloped, spherical capsid, capable of host genome integration. b. Electroporation-mediated CAR mRNA delivery Electric field-induced membrane permeabilization enables intracellular uptake of CAR-encoding mRNA, followed by ribosomal translation to produce CAR proteins. c. Transposon-based genomic integration CAR-containing donor DNA and transposase are co-delivered into immune cells. The transposase recognizes specific sequences on the donor DNA, excises CAR sequences, and then mediates their site-specific integration into the host genome via sequence recognition. d. Nanoparticle-mediated plasmid delivery Nanoparticles serve as carriers to encapsulate CAR-encoding plasmid DNA

expression in the mRNA-based strategy. Generally, vectors that do not integrate into the genome could permit transient expression of the inserted fragments, making certain viral vectors, such as adenoviruses and adeno-associated viruses, eligible candidates. One strategy facilitates transient CAR-T cell production in vivo following subcutaneous implantation by incorporating retroviral particles encoding CAR into scaffolds for T cell engineering, showing superior persistence compared to conventional CAR-T cells [280]. Non-viral vectors such as liposomes and nanoparticles exhibit comparable behavior in facilitating transient CAR expression. Injecting CAR-T cell-programmed nanoparticles represents a promising alternative to conventional CAR-T cell therapy, offering substantial benefits such as reduced costs, the capacity for large-scale production, and the potential to treat a broader patient population. Additionally, the in vivo programming approach obviates the need for invasive pre-treatment procedures, further enhancing patient convenience and safety. Transient CAR expression could be achieved by introducing inducible elements regulated by specific conditions, an approach known as "inducible CAR systems", which has been extensively employed in myriad studies to investigate various aspects of CAR dynamics and functionality. Using the CRISPR/Cas9 system to integrate the genes of CAR constructs into the downstream region of T-cell activation-dependent promoters correlates CAR expression with T-cell activation and generates a positive feedback loop that enables the control of CAR expression by modulating the cell activation state [281]. The drug-controlled VIPER CAR system devised by Li and colleagues, comprising both inducible ON and OFF switches, could tailor T cell activation levels in real-time and limit CRS, thereby obtaining a favorable safety profile [282]. Moreover, researchers illustrated the manipulability of the VIPER CAR system in xenotumor-transplanted mice, showing enhanced functionality when contrasted with alternative drug-gated systems.

Transient expression of co-stimulatory factor

Artificial gene circuits enable the transient expression of accessory molecules in an inducible manner, fueling the anti-tumor activity and reinforcing the potencies of CAR-T cell therapy [189, 283]. The Uni-Vect system provides a novel paradigm for achieving transient expression of soluble cofactors [284]. The conventional approach to achieving transient expression entails employing a dual viral vector system, wherein a constitutively expressed CAR construct and an inducibly expressed cofactor are expressed, respectively. Nevertheless, the dual viral vectors engender the formation of heterogeneous cell populations, which in turn increases the cost of cell sorting and limits technique applicability. In contrast, the single-vector Uni-Vect system optimizes the dual-vector system via incorporating the NFAT gene, which is a transcription factor dependent on TCR activation [284]. The Uni-Vect system initiates the expression of downstream auxiliary molecules upon TCR-mediated tumor signal recognition, consequently activating the NFAT pathway while simultaneously reducing the adverse effects associated with the constitutive expression of adjuvant molecules [284]. Smole et al. further fortified CAR-T cell functionality by loading diverse adjuvant molecules into the Uni-Vect system, emphasizing that the system could augment tumor-antagonizing responses through IL-12, mitigate CRS via IL-6α, and stimulate CAR-T cell proliferation through FOXO1 [284].

Gene editing technology

Iterative updates in gene editing technology facilitated the next generation of advanced engineered cells. Traditional genetic engineering involves introducing donor DNA with flanking sequences homologous to the target site to achieve homologous recombination (HR) [285]. The wider application of HR is constrained by its inefficiency in many organisms. Programmable nucleases in genome editing recognize and cleave specific DNA sequences, potentially producing double-strand breaks (DSBs), which are subsequently repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR), depending on the availability of a donor DNA template [286]. NHEJ-mediated repair requires no template and connections are random, whilst random insertions, introductions or nucleotide deletions at the break sites may result in the loss of function of corresponding proteins [287]. Consequently, NHEJ could alter specific genes to achieve gene knockouts, whereas HDR could integrate donor DNA into DSB sites with greater precision, thereby enabling more accurate gene editing [288].

Gene editing endonucleases, namely DSB inducers, are partitioned into meganucleases (MN), zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and clusters of regularly interspaced short palindromic repeats (CRISPR)-associated nucleases (Cas) based on structure. Genetic engineering modifies the specificity of MN, an endonuclease, by generating proteins that incorporate MN structural domains or by modifying protein residues within the DNA-binding domain. However, the paucity of natural MNs and the complexity and inefficiency of modifying MN specificity constrain MN application [289]. ZFN, a DNA-binding protein comprising at least three zinc finger domains that each recognize three bases and bind to DNA via a helix, could target unique DNA sites within a cell by designing two ZFNs to recognize complementary strands of the same double-stranded DNA [290]. Similar to ZFN, TALEN

employs a comparable methodology and serves as a competitive alternative, which is a fusion protein consisting of a transcription activator-like effector (TALE) and a FokI endonuclease [291, 292]. TALE contains multiple conserved repeat units with residues at positions 12 and 13 responsible for nucleotide recognition [292]. The ability to redirect TALE specificity by redesigning these residues is more straightforward than modifying ZFN, thereby making TALEN a more competitive candidate for gene editing applications [293]. Frustratingly, all these approaches rely on intricate protein engineering to target specific DNA sequences, and delivering the proteins into cells for multiplexed editing poses additional obstacles, which constrain their extensive use in genome manipulation [293].

In contrast to programmable nucleases that recognize target sequences through protein-DNA interactions, the CRISPR/Cas9 system in gene editing relies on RNA-guided nucleases. The CRISPR/Cas9 system has ushered in a significant advancement in biological research, characterized by reduced editing costs, enhanced editing flexibility, and efficiency. The CRISPR system generates pre-crRNA and tracrRNA, which form a complex with Cas9, and the spacer sequence within the crRNA guides Cas9 to cleave the target DNA, while modifying the target sequence simply requires adjusting the sgRNA sequence [294, 295]. PAM is a short sequence motif (approximately 2–5 bp) positioned adjacent to the crRNA target sequence on invading DNA, and plays a pivotal role in sgRNA-guided recognition and cleavage of target sequences by Cas9 [295]. The superior efficiency and accuracy of CRISPR/Cas9 targeting, coupled with its extensive range of targeting genes and versatility, offer robust technical support for achieving complicated gene editing.

The CRISPR/Cas technology shows bright therapeutic prospects in multifaceted disciplines, including the development of antimicrobial agents to target highly toxic and drug-resistant bacteria [296], correcting CFTR gene defects in cystic fibrosis patients [297], preventing muscular dystrophy [298], and treating viral infections [299, 300]. Employing gene editing technology in cancer treatment has increasingly become intertwined with immunotherapy, particularly in CAR-T cell production (Fig. 7B). Notably, the utilization of autologous T cells, currently the predominant method for CAR-T cell production, faces numerous challenges, such as a lengthy production process, the need for personalized customization, high treatment costs, and the lack of sufficient quality and quantity of autologous immune cells in many late-stage cancer patients due to prior radiation and chemotherapy [301]. Reassuringly, allogeneic donor T cells, an off-the-shelf product, serve like a precision scalpel, adeptly slicing through the intricate knots of autologous CAR-T cell production, providing an efficacious and generalized therapeutic strategy for patients [302]. However, allogeneic T cells expressing endogenous TCRs may recognize alloantigens in the recipient, leading to GvHD, and the host's immune system may also mount a rapid immune rejection against allogeneic T cells via potent immune responses to HLA molecules on their surface [303, 304]. Utilizing ZFN and TALEN technologies to knock out endogenous TCRs in T cells has been demonstrated to effectively prevent the occurrence of GvHD [305, 306], while the additional elimination of HLA-A molecules on allogeneic T cells could significantly mitigate host immune rejection [307]. TALEN technology has also been reported to eliminate TRAC, CD52, and deoxycytidine kinase from CAR-T cells to generate universal CAR-T cells with an efficacious tumor-inhibitory effect [308, 309]. Utilizing CRISPR/Cas9 to target and eliminate B2M and TRAC genes generates CAR-T cells with enhanced lytic activity and reduced adverse effects, providing a safer, more stable, and durable therapeutic option [310]. Nevertheless, the independent processes of silencing endogenous genes and integrating the CAR transgene lead to a heterogeneous population of CAR-T cells, characterized by variable levels of gene modification and CAR expression. Georgiadis et al. integrated CAR expression alongside CRISPR/Cas9-mediated gene editing via embedding sgRNA elements into the ΔU3 3′ long terminal repeat (LTR) of a CAR-encoding lentiviral vector, and the subsequent cell selection process, facilitated by magnetic beads, could effectively generate high levels of TCR^-/CAR^+ edited cell populations [311]. Additionally, the random integration of CAR transgenes into T cells poses potential risks, including oncogenic transformation, transcriptional silencing, and clonal expansion. To tackle these challenges, Eyquem et al. strategically directed the insertion of a CAR transgene to the T-cell receptor α constant (TRAC) locus, a genomic site that governs the expression of endogenous TCRs, thereby attaining consistent CAR expression while selectively diminishing TCR levels. This innovative strategy amplifies the efficacy of tumor rejection, curtails tonic CAR signaling, and postpones T-cell differentiation and exhaustion [312, 313]. The implementation of a multifaceted gene editing strategy, involving the incorporation of multiple gRNA expression cassettes into the lentiviral vectors of a single CAR, facilitates the generation of universal CAR-T cells with disruption of the inhibitory FAS, PD-1, and CTLA-4 pathways, hence augmenting their durability and effectiveness in combating tumors [314]. Furthermore, overexpression of HLA-E [315] and CD47 [316] has been demonstrated to attenuate host NK cell and macrophage-mediated immune responses.

In addition to generating universal CAR-T cells, a vast array of studies indicated that the targeted knockdown of inhibitory receptors or signaling molecules, including PD-1, CTLA-4, Fas, and others, could significantly augment the therapeutic efficacy and persistence of these cells. This strategy effectively enhanced the performance of immune cells in counteracting the immunosuppressive TME, thereby enhancing the overall efficacy of immunotherapy [317–319]. Further, CRISPR/Cas9 technology could edit up to five genes for efficient multiple-gene editing. The CRISPR/Cas9 technology illuminates the viability of comprehensive genome engineering in mice, unveiling new frontiers for exploring entire signaling pathways and genes with redundant functions [320]. Inspiringly, the first human experiment testing the safety of genome editing with the multiple CRISPR/Cas9 system showed the initial safety and feasibility of autologous TCR-T cells after simultaneous knockdown of TRAC, TRBC, and PD-1 in treating patients with advanced refractory tumors [321]. Notably, the removal of inhibitory molecules is a double-edged sword in immunotherapy. Accordingly, it is essential to examine whether the ablation of particular inhibitory signals may culminate in uncontrolled cell proliferation or severe autoimmune responses.

Vector-mediated gene delivery

Currently approved commercial CAR-T products deliver CAR transgenes using lentiviral or retroviral vectors (Fig. 7C), an approach that, while generally effective, presents certain non-negligible limitations: 1) Viral vectors could be constrained in their capacity to accommodate cargo of significant size (5–15 kbp) and exhibit notable cytotoxicity [194]; 2) Introducing genes from viruses into the human genome poses substantial safety concerns [322, 323]; 3) The need to accommodate an ocean of gene cargoes necessitates sophisticated vector engineering, whilst their manufacturing process is inherently costly. Various studies concentrate on exploring non-viral gene delivery methods to overcome safety and financial challenges associated with viral vectors. Electroporation utilizes high-voltage electric fields to generate transmembrane pores, thereby facilitating the permeation of macromolecular carriers into the cellular interior [324] (Fig. 7C). However, the potency of electroporation is contingent upon the transient expression of CAR, which persists for only a few weeks. Furthermore, robust electric fields have been demonstrated to precipitate cellular dysfunction, including lipid peroxidation, protein denaturation, the generation of reactive oxygen species, and DNA damage, which ultimately culminates in a reduction in cell viability [325]. The relative simplicity, low cost, and suitability for mass production of transposon vectors have led multiple researchers to endeavor to investigate transposon-mediated gene delivery platforms such as Sleeping Beauty and PiggyBac systems [326, 327] (Fig. 7C). Despite their relatively diminished transduction efficiency compared to viral systems, they are perceived as a safer alternative. Nevertheless, malignant transformation of CAR-T cells was reported in two patients with B-cell malignancy treated with CD19-specific CAR-T cells generated via the PiggyBac transposon platform, indicating that the transposon system also possesses potential risks [328].

The feasibility and high efficiency of the CRISPR/Cas9 gene delivery strategy have been empirically validated in human cells, thereby establishing a crucial scientific foundation for accelerating the advancement of gene therapy and precision medicine. The CRISPR/Cas9 genome-targeting system could successfully correct ILR2A mutations in patients with autoimmune diseases [329], restoring cell signaling functions [329]. Additionally, the CRISPR/Cas9 genome-targeting system could replace endogenous TCRs with novel TCRs, thereby generating engineered TCR-T cells that exhibit precise retargeting to TAAs and display highly efficacious antitumor functionalities. However, knock-in targeted by the CRISPR/Cas9 system presents potential toxicity of the DNA template used, and evidenced less efficiency than AAV-mediated gene delivery [330]. Donor DNA delivery based on CRISPR/Cas9 technology, by incorporating CD19/TAG-72 scFvs into CD3ε to generate T cells expressing fusion proteins, could enhance tumor-targeting capacity and even demonstrate more efficacious oncolytic potential than CAR-T cells, in Raji cell xenograft models [331].

Using biochemical carriers, such as detergents and pore-forming toxins, enables the penetration of cell membranes to form pores that facilitate cargo passage [332] (Fig. 7C). Nevertheless, biochemical carriers also encounter significant obstacles, including incompatibility of cargo materials with carriers, which could be influenced by factors such as charge, size, and hydrophobicity; limited compatibility of carrier materials with target cells, which may hinder efficient endocytosis; and inherent potential toxicity.

Nanoparticles, which belong to a magnetic category of delivery vehicles, offer a panoply of substantial merits, including enhanced safety, efficiency, and controlled pharmacokinetic properties (Fig. 7C). The distinctive physical, chemical, and biological characteristics of nanoparticles have rendered nanoparticles a subject of interest across diverse disciplines, involving medicine, electronics, and the environment. Nanoparticles could load reagents and facilitate their targeted delivery to specific sites, navigating restrictions imposed by physiological

mechanisms. Therefore, nanoparticle utilization in CAR-T cell production portrays an alternative, off-the-shelf product that is distinct from universal CAR-T cells, specifically enabling in vivo CAR-T cell generation [333]. In contrast to other delivery technologies, nanoparticle-mediated delivery doesn't require mechanical permeabilization of the cell membrane and could bind to receptors on target cells to initiate endocytosis for cargo delivery. Following administration, nanoparticles could continuously program effector cells to generate sufficient quantities of CAR-T cells, which elicit vigorous immunity responses comparable to those of commercial CAR-T cell products [334].

Compared to the labor-intensive and complex ex vivo production employing traditional viral vectors, the nanoparticle-mediated generation of CAR-T cells depicts a novel strategy. The nanoparticle-mediated generation of CAR-T cells necessitates only a few straightforward steps and could be manufactured at multiple centers, thus offering significant economic benefits. Notably, generating CAR-T cells in vivo is particularly challenging because it requires reprogramming senescent and depleted T cells, which are often compromised in cancer patients. Such impairment, frequently a result of extensive pretreatment, can significantly undermine the efficiency of the reprogramming process. By loading nanocarriers with TCR-stimulating signals or pro-cell growth factors, transduced T cells exhibit boosted survival and proliferation [335]. The co-delivery of anti-programmed cell death-1 (anti-PD1) and anti-tumor necrosis factor receptor superfamily member 4 (anti-OX40) via dual immunotherapy nanoparticles has been demonstrated to induce a more pronounced T cell activation alongside an enhanced immunological memory, consequently exhibiting markedly superior therapeutic outcomes [336]. Lipid nanocarriers epitomize a gold standard for non-viral gene delivery vectors. In the heart failure mouse models, CD5-targeted lipid nanoparticles could effectively deliver modified mRNA to T cells, thereby achieving transient and efficient expression of CD19-CAR [337]. By devising activated lipid nanoparticles (aLNPs) that mimic antigen-presenting cells (APCs), Metzloff et al. achieved one-step activation and transfection of primary human T cells [338]. This reduces the complexity of producing CAR-T cells [338]. Additionally, injecting aLNPs effectively decreased tumor size in the murine xenograft model of leukemia, showing their potential as a fast and efficient platform for making mRNA CAR-T cells [338].

Perspective and outlook

Considering the prevailing complexities associated with immunotherapy, the selection of strategies necessitates a meticulous evaluation of existing impediments, particularly in the context of solid tumor treatment, wherein tumor heterogeneity and off-target toxicity emerge as predominant considerations.

The AND-gate endeavors to partially emulate the targeting of TSAs through integrating diverse TAAs, thereby augmenting precise antigen-specific recognition. Additionally, precise tumor targeting offers the potential benefit of reducing non-specific immune activation, thereby reducing the risk of exhaustion. Nevertheless, this strategy introduces a dual-edged effect: while it enhances the safety profile of targeting mechanisms, the AND-gate imposes more stringent requirements on the co-expression levels of antigens on the tumor surface, thereby posing a potential risk of CAR-immune cell activation failure due to the downregulation or complete loss of expression of a single antigen. OR-gate aims to effectively broaden the coverage of tumor heterogeneity by integrating diverse TAA targets. However, this strategy faces the challenge of rigorously selecting target combinations to balance therapeutic efficacy with potential off-target risks. The ultimate efficacy of the OR-gate is contingent upon the quality of the selected TAA combinations. In the absence of rigorous preclinical validation and systematic design, indiscriminate stacking of multiple TAAs may exacerbate the risks of off-target effects and non-tumor toxicity [339, 340]. Moreover, broader tumor targeting of the OR-gate might induce heightened immune stress, potentially increasing the risk of tumor immune escape and susceptibility to self-exhaustion [339]. NOT-gate improves safety by excluding certain normal tissues from targeting, which is valuable in specific situations where there are well-defined 'safety antigens' in key normal tissues. Simultaneously, NOT-gate expands the targetable antigen range. The challenge, however, resides in identifying and validating suitable and broadly applicable safety antigens, coupled with the necessity to meticulously balance activation and inhibition signaling to ensure optimal functional efficacy. SUPRA CAR presents advantages with a dose-adjustable safety profile, offering precise modulation of efficacy and safety, although its clinical value awaits further validation through additional data.

Multi-specific antibodies and cell engagers embody a distinct therapeutic paradigm compared to gene-edited immune cells. Multi-specific antibodies and cell engagers, with several FDA-approved products currently available [264], signify notable advancements in immunotherapy, particularly in the short term and for specific tumor types. The efficacy of a multi-specific antibody/cell engager, whether functioning as a bridge between tumor cells and immune cells or blocking inhibitory signals, is closely associated with the functional status of the patient's intrinsic immune system. In contrast,

gene-edited immune cells function as "living drugs" engineered to exert a more proactive tumoricidal effect, potentially eliciting distinct therapeutic responses and exploring the possibility of achieving profound, durable remissions. Although gene-edited immune cells and multi-specific antibodies/cell engagers have demonstrated substantial efficacy in treating hematological malignancies, within the more complex domain of solid tumor therapy, they encounter shared challenges of the TME. BsAb/BiTE were designed to target specific components/immune checkpoints within the TME to counteract the immunosuppressive effects of the TME and enhance therapeutic efficacy [341–343]. Conversely, gene-edited immune cells tend to address the complexity of the TME by reprogramming cellular functions. For instance, CAR-M cells aim to infiltrate and remodel the TME. In addition, CAR-T cells are being engineered with logic gates (e.g., targeting the TME cytokine—TGFβ/IL-4) and other modifications specifically designed to counteract the TME. In efforts to augment persistence and mitigate exhaustion, CAR-immune cells have undergone genetic modifications to enhance their intrinsic durability, metabolic adaptability, and resistance to exhaustion [344, 345]. Whereas the action duration of multi-specific antibodies/cell engagers is mainly influenced by pharmacokinetics [346]. However, by exploring strategies such as targeting costimulatory receptors, there is potential to endow multi-specific antibodies/cell engagers with enhanced and prolonged immune responses [347]. Finally, in terms of production and application, multi-specific antibodies/cell engagers often benefit from relatively mature manufacturing processes and their "off-the-shelf" potential. In contrast, CAR-immune cells face complex production and high cost limitations. However, their prospects are substantial, with advancements like allogeneic CAR therapies, in vivo CAR-T cell generation, and non-viral methods reducing complexity and costs, improving future application convenience.

Future development of multi-targeting designs relies on further optimization of CRA/antibody structure, balance of multi-gene co-expression, screening for better targets, and facilitating clinical translation (automated production, efficacy prediction). AI empowers the development of these strategies. The advancements in artificial intelligence, a focal point of the 2024 Nobel Prize, could drive CAR-immune cell development in multiple ways. In clinical translation, AI analyzes patients'baseline information, genomic, and proteomic data. The synergy of AI with Internet of Things (IoT) sensors streamlines the complex process of CAR-immune cell production and monitors the conditions during transportation and storage (Fig. 8).

By analyzing patients'immune status, tumor characteristics, and genetic information, AI could screen suitable candidates for various gene-edited cell therapies and personalize treatment plans while providing predictions of treatment outcomes [348–350]. Ongoing surveillance of patients'physiological parameters and relevant biomarkers using AI helps in the early identification of potential adverse reactions, thereby enabling timely intervention and enhancing the safety of immunotherapy. Additionally, by integrating large-scale data analysis from scientific literature, AI could drive the structural optimization of CAR constructs and the discovery of new targets for immunotherapy [350]. The harmonious combination of AI with CRISPR/Cas9 technology enables precise identification of genetic targets, predicts, the prediction and minimization of off-target effects, and the optimization of guide RNA designs, thereby enhancing the specificity of CRISPR/Cas9 technology [351].

The determination of antibody-antigen recognition specificity is critical for immunotherapy, whereas conventional approaches frequently encounter limitations, including high costs and long experimental durations. Current computational models exhibit significant dependence on 3D structural data, yet technical barriers in data acquisition constrain their biological applications [352, 353]. Yan Huang et al. developed AbAgIntPre, an innovative deep learning framework that exclusively utilizes amino acid sequence data for antibody-antigen interaction prediction. The model was trained on high-quality antibody-antigen complexes obtained from SAbDab (a database that collects the structures of antibody and antigen complexes) and CoV-AbDab (a database that specializes in collecting data on antibodies that bind to coronaviruses), enabling broad-spectrum antigen interaction prediction [354]. Expectedly, this model has considerable potential for CAR target screening by sequence prediction of antibody-antigen binding, allowing rapid screening of high-affinity antibody fragments for CAR construction. This computational framework demonstrates significant potential for CAR target screening through sequence-based prediction of antibody-antigen binding affinities, enabling systematic identification of high-affinity antibody fragments critical for chimeric antigen receptor engineering. In addition, the SARS-CoV model constructed by Huang et al. validated the ability to predict mutant antigens [354]. This feature might be used for CAR design of new epitopes or mutant proteins targeting tumor antigens, bridging the bottleneck of the lack of structural data for tumor neoantigens. Though modelling patient-specific antigen binding, AbAgIntPre is expected to support individualized CAR design, thereby reducing the risk of off-target effects.

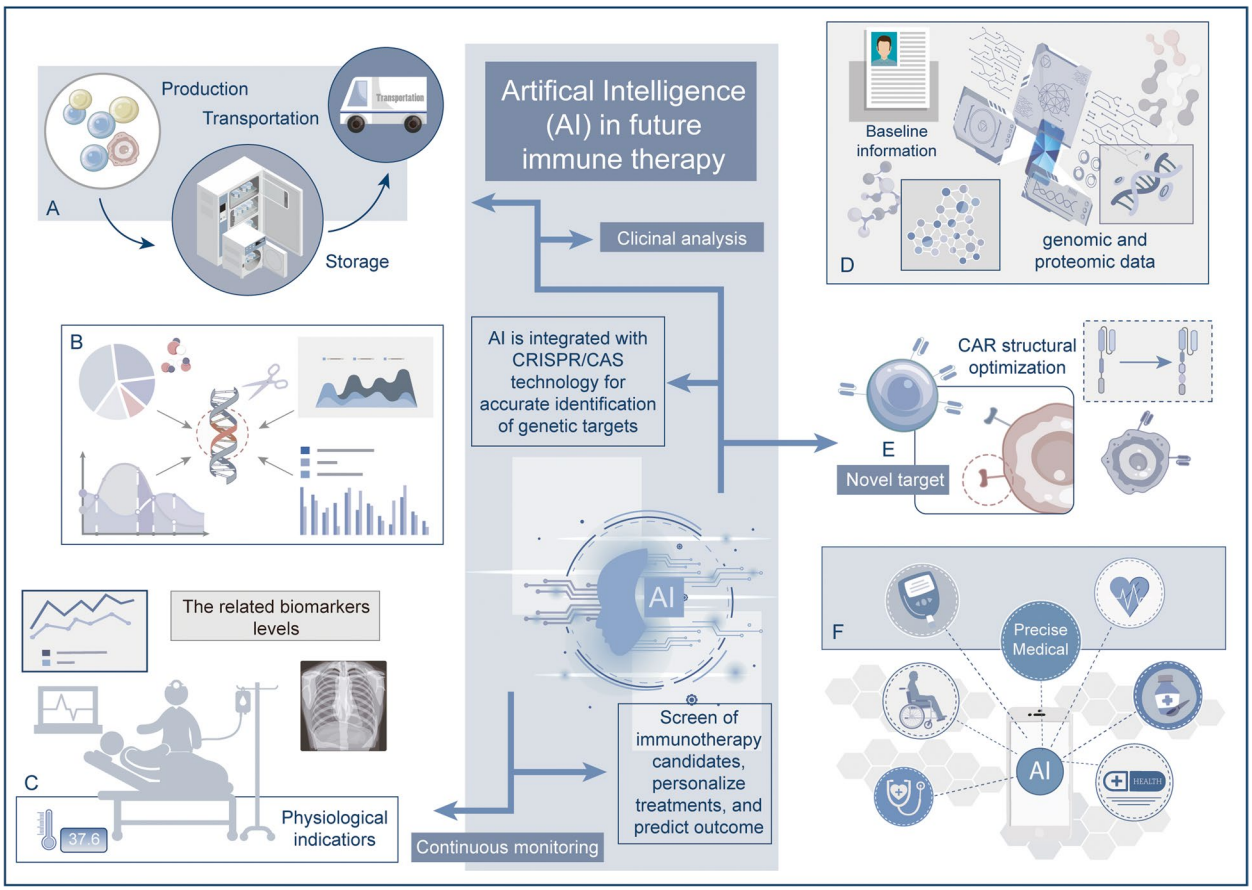

Fig. 8 Application of Artificial Intelligence (AI) in Immunotherapy. **A**. AI plays a pivotal role in the production, storage, and logistics of CAR-immune cells by leveraging IoT sensors. **B**. AI enhances the precise identification of CAR targets through integration with CRISPR/Cas9 technology. **C**. AI continuously monitors immunotherapy patients via the detection of biochemical indicators. **D**. AI is capable of analyzing baseline information, as well as genomic and proteomic data from patients. **E**. AI facilitates the discovery of novel targets and the optimization of CAR construct structures. **F**. AI can screen suitable candidates for various gene-edited cell therapies, develop personalized treatment plans, and predict treatment outcomes

In CAR-T cell therapy, structural optimization of scFv constitutes a pivotal step in augmenting therapeutic effectiveness. Martarelli et al. implemented AI-driven computational platforms to perform molecular docking and targeted molecular dynamics analysis of disparate anti-CD30 monoclonal antibodies, enabling systematic screening of optimal scFvs for the construction of CARs. This approach exemplifies the potential of computational modeling platforms to catalyze the engineering of novel CAR constructs, while concomitantly minimizing financial expenditures and conserving resources [355]. CAR-Toner, an AI-based tool, could predict and optimize the tonic signaling of CAR-T cells, thereby improving their persistence and promoting expansion [356]. By integrating protein databases, structural biology, and advanced deep learning models, the CAR-Toner platform has been pre-trained on over 60 million protein sequences, which could calculate the positive charge patch (PCP) score of proteins and provide strategies to optimize the PCP score through induced point mutations. In the CLL1 antibody refinement, CAR-Toner analysis revealed that a series of CLL1 antibody variants generated by systematically reducing the PCP score eliminate tumors more efficiently compared to wild-type CARs [356]. Additionally, AttAB-seq represents a sequence-based computational platform that predicts alterations in antigen-antibody binding affinity, occasioned by antibody mutations, thus offering a novel strategy for antibody optimization [357]. Jin and colleagues employed AttABseq to determine optimization trajectories for SARS-CoV-2 VHH and Ebola virus antibodies, demonstrating strong concordance between computational predictions and experimental measurements that validated the platform's reliability [357]. This achievement provides a valuable lesson for CAR scFv

affinity optimization. However, implementation necessitates integration of supplementary datasets (including tumor antigen-specific mutant binding profiles) and experimental validation in oncology field.

Furthermore, AI has demonstrated transformative potential in CAR antigen prediction and target optimization. Mason et al. developed an integrated framework combining CRISPR-Cas9-mediated antibody mutant library and training convolutional neural network-based antigen specificity prediction models, enabling high-throughput identification of functionally optimized therapeutic antibody variants from a multitude of sequences [358]. Additionally, machine learning models could predict and validate optimal metabolic pathways, thereby optimizing the metabolic processes of CAR-T cells and enhancing their efficacy against solid tumors, leading to metabolically enhanced CAR-T cell therapy products [359]. Employing nanogrid-based time-lapse imaging combined with visual artificial intelligence, Rezvan et al. identified a subpopulation of CD8-fit T cells with high migratory and continuous killing capabilities, which are primed to boost cancer treatment outcomes in CAR-T therapy [360].

Notably, in the early stages of AI applications in immunotherapy, the lack of appropriate datasets poses significant challenges for training initial machine learning algorithms. The complexity of various biological structures and patient information further complicates these efforts, which necessitates continued exploration. Nevertheless, the comprehensive AI platform development, from antigen selection to CAR-T cell structure optimization, production, clinical trials, and patient prediction, through the integrated synergy of multi-omics data such as genomics, proteomics, and imaging, will contribute to more precise gene-edited immune cell therapies [350].

Conclusions

While gene-edited immune cell therapies, particularly CAR-T cells, have achieved remarkable success in hematological malignancies, realizing their full potential as next-generation immunotherapy tools, especially in solid tumors, requires overcoming significant challenges. To surmount the limitations of CAR-T cells, extensive studies prioritize developing alternative gene-edited immune cells such as CAR-NK and CAR-M cells. Furthermore, harnessing multi-targeting strategies havs emerged as a critical direction to broaden targetable antigens, counteract tumor heterogeneity, and improve clinical efficacy. Innovative technologies like Boolean logic gates, SUPRA CARs, multi-specific antibodies, and cell engagers are accelerating the development of multi-targeting immunotherapies by providing enhanced flexibility, improved specificity, and simplified design frameworks for diverse targeting applications.

Progression in next-generation immunotherapy tools is being propelled by emerging technologies in gene editing and gene delivery. Synthetic mRNA and transient modification methods, CRISPR/Cas9-mediated cell engineering, and novel synthetic materials for gene delivery provide a boost to enhance tumor susceptibility to immunotherapies and achieve sustained specific-targeting capabilities of engineered immune cells. Looking ahead, the integration of AI holds immense promise in further optimizing these next-generation immunotherapy tools. AI-driven approaches can revolutionize target identification, CAR design, and personalized treatment strategies, paving the way for more effective and safer immunotherapies.

The future of next-generation immunotherapy is bright, with the convergence of tumor analysis, synthetic biology, and artificial intelligence promising to unlock even more sophisticated and effective therapeutic strategies [339]. Harnessing multi-targeting strategies in conjunction with these advancements holds the key to realizing the full therapeutic prospects of gene-edited immune cells and transforming cancer treatment. Strategically oriented towards the future, continued development and combined application of gene editing technologies, gene delivery strategies, and biomaterials will offer robust backing to support and optimize the breadth and security of targeting profiles. Diverse disciplines could combine fully exploit the therapeutic potential of engineered T cells, sparking activation and amplifying their potency. The integration of metabolic engineering and CAR-T cells represents a novel synergistic approach to regulate CAR-T cell persistence through epigenetic and phenotypic modifications, which may emerge as a promising avenue for future research. The synergistic alliance of oncolytic viruses and CAR-T cells unveils a strategic approach for confronting the dilemmas of solid tumors (NCT05393440), encompassing oncolytic viruses encoding BiTEs, cytokines, and immune checkpoint inhibitors [134, 361], as well as oncolytic viruses encoding CAR targets [362, 363]. Moreover, as data sources from human trials continue to be refine, machine learning may assume a crucial role in the future advancement of novel therapies, facilitating the prediction of optimal parameters for critical treatment factors. The convergence of tumor analysis and synthetic biology may spur sophisticated paradigm innovation for tumor antigen identification, empowering more effective responses to the complex challenges inherent in tumor therapy.

Box 1. Key Terms

1. MHC: Cell-surface proteins required for antigen presentation to T cells. CAR-T cell therapies bypass MHC dependency to directly target tumors

2. CD19/BCMA: CD19, B-cell surface marker targeted in leukemia/lymphoma therapies. BCMA: Plasma cell marker in multiple myeloma. In CAR-T cell therapy, CD19/BCMA are important targets, and their loss is an important cause of tumor recurrence

3. Antigen-negative clone: Tumor subpopulations evade CAR-T cell recognition by downregulating target antigens, resulting in poor therapeutic efficacy

4. CRES: Possible neurological side effects of CAR-T therapy, such as confusion or seizures

5. CRS: A systemic inflammatory response triggered by the rapid release of cytokines from activated immune cells is a common side effect of CAR-T therapy

6. Solid tumor: Cancers that form solid masses, such as lung or breast cancer, as distinguished from hematologic malignancies

7. Autologous/Allogeneic cell therapies: Patient/healthy donor-derived cells (e.g., T cells) engineered ex vivo and reinfused into the same individual/patients

8. Universal allogeneic CAR-immune cell platforms: Standardized CAR-immune cell therapies developed from healthy donors are available for multiple patients

9. Off-target toxicity: CAR-T cell therapy targets healthy cells/tissues and causes damage

10. Adaptor: Molecules that act as connecting bridges to enhance the adaptability and specificity of CAR-immune cells

11. Multitargeting: Enables immune cells to attack multiple antigens simultaneously, distinguishing it from single-targeting strategies and reducing the risk of cancer escape due to antigen loss

Abbreviations

CAR	Chimeric antigen receptor
PBMC	Peripheral blood mononuclear cells
HSPCs	Hematopoietic stem cells
iPSCs	Induced pluripotent stem cells
FDA	Food and Drug Administration
R/R	Recurrent/refractory
BCMA	B-cell maturation antigen
CRS	Cytokine release syndrome
CRES	CAR-T cell-related encephalopathy syndrome
AI	Artificial intelligence
GvHD	Graft-versus-host disease
CAR-NK	CAR-natural killer
CAR-M	CAR-Macrophage
TCR	T cell antigen receptor
pMHC	Peptide-major histocompatibility complex
scFv	Single-chain fragment variable
VL	Variable regions of monoclonal antibody light
VH	Variable regions of monoclonal antibody heavy
CD	Cluster of differentiation
ICOS	Inducible co-stimulator
ITAMs	Immunoreceptor tyrosinebased activation motifs
APC	Antigen-presenting cell
TRUCKs	T-cells redirected for universal cytokine-mediated killing
NK	Natural killer
ADCC	Antibody-dependent cell cytotoxicity
DAP12	DNAX-activation protein 12
TME	Tumor microenvironment
MMP	Matrix metalloproteinases
TAM	Tumor-associated macrophage
NKT	Natural killer T
MAIT	Mucosa-associated invariant T
ECM	Extracellular matrix
MRD	Minimal residual disease
CR	Complete response
PFS	Progression-free survival
LBCL	Large B-cell lymphoma
DIPG	Diffuse intrinsic pontine glioma
DNR	Dominant-negative receptors
ALK	Anaplastic lymphoma kinase
MUC1	Mucin 1
TAA	Tumor-associated antigen
TSA	Tumor-specific antigen
MART-1	Melanoma antigen recognized by T cells 1
CEA	Carcinoembryonic antigen
NY-ESO-1	New York esophageal squamous cell carcinoma-1
ICANS	Immune effector cell-associated neurotoxicity syndrome
HPSC	Human pluripotent stem cell
TruCs	T cell receptor fusion constructs
HER2	Human epidermal growth factor receptor 2
IL13Rα2	Interleukin-13 receptor subunit alpha-2
EphA2	Ephrin-A2
GBM	Glioblastoma
ALPPL2	Alkaline Phosphatase Placental-like 2
SNIPRs	Synthetic intramembrane proteolysis receptors
AML	Acute myeloid leukemia
ITIM	Immunoreceptor tyrosine-based inhibitory motifs
NASCAR	Neoplasm-targeting allele-sensing CAR
CEA	Carcinoembryonic antigen
LIR-1	Leukocyte Ig-like receptor 1
SUPRA	Split, universal, and programmable
BsAbs	Bispecific antibodies
FcR	Fc receptor
DVD-Ig	Double variable domain Ig
DAF	Double-acting Fab
DNL	Docking and locking
TsAb	Tri-specific antibody
BiTEs	Bispecific T cell engagers
BLA	Biologics license application
IFN-γ	Interferon-gamma
EpCAM	Epithelial cell adhesion molecule
TriTEs	Tri-specific T cell engagers
CRC	Colorectal cancer
HSA	Human serum albumin
CLDN18.2	Claudin 18.2
NSCLC	Non-small cell lung cancer
CiTE	Checkpoint inhibitory T-cell-engaging
TriKEs	Tri-specific Killer Engagers
CLL	Chronic lymphocytic leukemia
CLEC12 A	C-type lectin domain family 12 member A
HR	Homologous recombination
DSB	Double-strand breaks
NHEJ	By non-homologous end joining
HDR	Homology-directed repair
MN	Meganucleases
ZFN	Zinc finger nucleases
TALEN	Transcription activator-like effector nucleases
CRISPR	Clusters of regularly interspaced short palindromic repeats
Cas	Associated nucleases
TALE	Transcription activator-like effector
sgRNA	Single guide RNA
PAM	Protospacer adjacent motif
TRAC	T-cell receptor α constant
aPD1	Anti-programmed cell death-1
aOX40	Anti-tumor necrosis factor receptor superfamily member 4

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Authors' contributions
QL and RX provided direction and guidance throughout the preparation of this manuscript. HZQ and ZKZ wrote and edited the manuscript. ZKZ reviewed and made significant revisions to the manuscript. HZQ, RS, YMM, YDX, PF, GYC, PPZ, WJC, YC and YJC collected and prepared the related papers. All authors read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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